The molecular biology of tolerance to *Bacillus thuringiensis* endotoxin in *Helicoverpa armigera*: a novel mechanism and its genetic transmission

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To my parents, my wife and my son.

Table of contents

Statement

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August 2005

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Abstract

Resistance in insect pests against the endotoxin of *Bacillus thuringiensis* (Berliner) (Bt) is a major threat to the usefulness of this biopesticide both used as traditional formulations and in transgenic crops. A crucial requirement for the development of successful resistance management strategies is a molecular understanding of the nature and inheritance of resistance mechanisms. This information can be used to design management strategies that will delay or counteract Bt resistance.

Most known Bt resistance mechanisms in field or laboratory-selected populations are recessive mutations based on receptor inactivation. However, there are occasional examples of dominant or semi-dominant mechanisms with unusual characteristics. For example, Bt-resistance was selected in a *H. armigera* population by feeding the toxin with artificial diet in the laboratory. The resulting resistance was based on a single gene locus with a semi-dominant trait. This population was backcrossed with a susceptible strain to get nearly isogenic resistant and susceptible lines. After four crosses and selection for resistance, the resulting ISOC4 strain was analysed for Cry1Ac binding to BBMVs. This assay showed differences in binding to an unknown protein, while none of the known aminopeptidase N genes showed any mutation that could be responsible for the lack of toxin binding. Moreover, exposure to Bt toxin imposed a fitness cost, which is likely to derive from pleiotropic effects of the resistance gene rather than effects from other gene loci. This suggested that resistance was probably not based exclusively on the inactivation of one of the major receptor genes but on effects from (an) other unknown gene(s), which also affected developmental functions.

A melanization reaction occurs in the hemolymph and gut lumen of the ISOC4 strain, which may be associated with an elevated immune status in the resistant strain. Further analysis showed that melanization in cell-free hemolymph (plasma) and gut was significantly higher in the ISOC4 than in the susceptible ANGR strain. This is associated with a higher coagulation reaction in the ISOC4 strain, which precluded conventional protein analysis using SDS-PAGE. To study the immune induction in this strain and the effects of an elevated immune status on Bt resistance, sub-lethal doses of the Bt–toxin, Cry1Ac, were fed with the artificial diet. Under these conditions the effects of immune induction were studied in ANGR larvae. It was found that the Bt feeding resulted in the induction of an 85 kDa hemolymph protein.

Analysis of the native p85 was performed using a coagulation bioassay of hemolymph plasma. This showed that p85 specifically interacts with Cry1Ac and other oligomeric lectins, suggesting that p85 is an immune-related protein. To determine the identity of p85, an in-gel protein digest, or and peptide sequence analysis were performed to determine approprate primers for PCR amplification of the gene. The DNA product of the PCR reaction was sequenced and showed strong similarity to hexamerin from *H. virescens.* Antibodies created against a recombinant peptide from the *H. armigera* hexamerin were obtained in rabbits and recognised p85 on Western blots. Using the anti-hexamerin antibodies, hexamerin was found in the gut lumen in addition to the hemolymph. In protein extracts from ISOC4 gut preparations, multiple bands of hexamerin are detected, which may be caused by proteolytic digestion or post-translational modifications. This suggests that hexamerin has a dual function. Apart from its metabolic function as a storage protein, it also

takes part in coagulation reactions, which is consistent with similar findings in *Drosophila melanogaster.*

Since hexamerin may have been affected in the ISOC4 strain by post-translational modifications, altered glycosylation in the resistant strain may directly or indirectly occur as a result of the elevated immune-status. Anti-glycosyltransferase antibodies from nematodes were examined for possible cross-reactivity to insect glycosyltransferase (Gly-T). In Western blots from gut extracts the anti-glycosyltransferase antibodies reacted with both ISOC4 and ANGR extracts, but binding was stronger with the ANGR extracts. In addition, Gly-T activity was examined in BBMVs from the gut and found to be different in ISOC4 and ANGR. Since Gly-T can transfer sugars onto proteins or lipids, it may modify hexamerin or associated lipids thereby changing hexamerin aggregation in the gut lumen. Since altered coagulation is a dominant trait, the observation that resistant *H. armigera* had a reduced Gly-T activity, is compatible with a change in post-translational modification of gut proteins and lipids in the ISOC4 strain.

If the tolerance to Bt toxin is caused by a transient induction of the immune system, the effect should not be visible in the next generation. However, since hexamerin is a storage protein and incorporated into oocytes during oogenesis, it is possible for a modified hexamerin to stimulate the immune system in the embryo, or directly sequester the toxin in the embryonic gut by a coagulation reaction, where it may protect the emerging neonates against the toxin. If this is correct, the neonates will only be protected if they derived from a resistant mother, whereas the offspring from reciprocal crosses will be susceptible. To test this assumption, bioassays were

performed on the progeny of the two crosses and survival of neonates examined. When resistant females were mated with susceptible males, the offspring showed higher tolerance to Cry1Ac compared to offspring from the reciprocal cross. Furthermore, the tolerance was not sex-linked. This suggests that the Bt tolerance in ISOC4 is transmitted via a maternal effect when *H. armigera* larvae were subjected to relatively low dosage of Cry1Ac toxin. However, the results from the two reciprocal crosses also showed that embryonic traits (nuclear genes) might also contribute to the Bt resistance.

When Western blots of gut protein extracts were developed with alkaline phosphatase reagents in the absence of the antibodies, a stained band was detected in ANGR extracts, which was significantly reduced in ISOC4 extracts. This chance observation suggests that a gut alkaline phosphatase, which retains enzymatic activity after SDS-PAGE, is significantly reduced in the resistant strain. Microsequencing of this band revealed four peptides, which all have similarity to a membrane-bound *Bombyx mori* alkaline phosphatase. Since alkaline phosphatase proteins are known bind to Cry1Ac, a genetic alteration in the ISOC4 strain may account for the recessive trait of Bt resistance at high dosage, which did not show a maternal effect. The role of the alkaline phosphatase protein in the cause of resistance in the ISOC4 strain remains to be elucidated. The corresponding gene may carry a mutation and thus represent the recessive gene locus assumed to be responsible for the phenotype. Alternatively, gene expression may be affected by another unknown gene as a result of the elevated immune status in the ISOC4 strain.

In summary, the observed alterations in the ISOC4 strain are compatible with a complex phenotype consisting of two components, a recessive mutation in an unknown gene, which could either be a Bt-receptor such as alkaline phosphatase and a dominant trait that appears to operate through an elevated immune status.

Chapter 1: Literature review

1.1 Introduction

More than hundred years of Bt toxin application have also witnessed a rapid period of discovery of new toxins (Table 1-1). The potency and specificity of Bt cry toxins for their insect targets make them ideal for control of insect pests of agricultural and medical importance (Betz et al., 2000). Bt toxins are now widely used for pest control in the forms of Bt formulations and transgenic Bt crops (Ferre and Van Rie, 2002; Shelton et al., 2002). A number of cry toxin genes have been engineered into important crop plants, such as corn, cotton and rice (Babu et al., 2003). According to (Bates et al., 2005) the accumulated area for Bt transgenic crops is presently about 80 million hectares. The large areas planted with Bt crops or treated with Bt sprays exert significant selection pressures on insect pests for the evolution of Bt resistance.

1.2 Literature review

1.2.1 Bt toxins

Bt toxins are produced by the soil borne and gram-positive bacterium *B. thuringiensis* (Bt). Bt has a wide range of hosts and is found in almost every ecological niche constituted by invertebrates. *B. thuringiensis* can accumulate large quantities of insecticidal toxins—δ-endotoxins, which are produced in so-called mother cells during the stationary phase (Agaisse and Lereclus, 1995). The parasporal crystals contain potent insecticidal delta endotoxins. They have been divided into two groups on the basis of their target specificity: the insect-specific cry proteins and the generally cytolytic cyt proteins (Schnepf et al., 1998). The structure of cyt crystal is significantly different from that of cry toxins. They only have one single domain and

it is the β-strands rather than the α-helix that is inserted into the cell to form a pore (Butko, 2003; Li et al., 1996).

Table 1-1 Brief history of Bt toxin use

Year	Country	Events
1901	Japan	Shigetane Ishiwata first isolated and named *Bacillus sotto* (Ishwata, 1901)
1911	Germany	Ernst Berliner re-discovered and re-named *Bacillus thuringiensis* (Berliner, 1911)
1915	Germany	Ernst Berliner reported the Crystal in Bt (http://www.bt.ucsd.edu/bt_history.html)
1938	France	Commercialised spore based formulations called Sporine (Worthington, 1991)
1955		Correlated insecticidal activity with the parasporal crystal.(Hannay and Fitz-James, 1955)
1958	US	*Bt* was used commercially (Ghassemi, 1981)
1961	US	*Bt* was registered as a pesticide with the EPA.
1977		The first subspecies toxic to dipteran (flies) species was found (Goldberg and Margalit, 1977)
1980's		Use of *Bt* increased when insects became increasingly resistant to synthetic insecticides and scientists and environmentalists became aware that the chemicals were harming the environment (Betz et al., 2000)
1981	US	*Bt* cry toxin gene was transferred and expressed into *E. coli* (Schnepf and Whiteley, 1981)
1983		The first discovery of strains toxic to species of coleopteran (Krieg et al., 1983)
1985	US	First report of Bt resistance in *P.interpunctulla* (McGaughey, 1985)
1987		Use of transgenic plants (Tobacco) to protect from insect attack (Vaeck et al., 1987)
1994	US	Bt resistance in *P xyllostella* was found in the field (Tabashnik, 1994)
1995	US	First Bt corn was registered by EPA (Shelton et al., 2002)
1996	US	Bt corn was commercially introduced (Shelton et al., 2002)
1996	US & Australia	Bt cotton commercialised
2004		Global areas for accumulated Bt crops since 1996 up to 80 million hectares (Bates et al., 2005)

Compared with the cyt crystal structure, cry-toxins have a distinct three-domain structure, where the amphipathic α helix inserts into the membrane to form a pore (Li et al., 1991). Currently (May 2005), about 311 different cry genes have been identified and sequenced (Crickmore et al., 2005).

Bt cry protoxins are 130 to 140 kDa proteins in which the C-terminal regions are highly conserved and are assumed to act as the crystallisation domain. After proteolysis in the midgut, it becomes a 60 to 65 kDa active toxin (Hofte and Whiteley, 1989). Cry toxins have a unique three-domain structure. Results of X-ray crystallography of the coleoptera-specific toxin Cry3A (Li et al., 1991) (Fig.1-1), lepidopteran toxins Cry1Aa (Grochulski et al., 1995), and Cry2Aa structure (Morse et al., 2001) showed that the cry toxins comprise a seven-helix bundle (domain I), a three-sheet β-strand domain (domain II) and a β-sandwich (domain III).

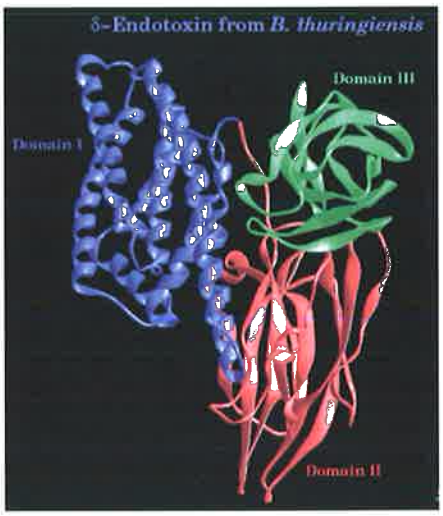

Fig.1-1. Crystal structure of Cry3A. The blue part is domain I which has 7 α helixes; the red part shows domain II with β-sheets; and the green part is domain III with β strands (Li et al., 1991).

Domain I is assumed to be a membrane insertion/toxicity region (Arti et al., 1999; Kumar and Aronson, 1999; Tigue et al., 2001). The six helices will facilitate the loop-insertion and consequently affect the pore-formation in the gut membrane

(Grochulski et al., 1995). Domain II consists of three antiparallel β-sheets, which play a key role in the receptor binding and its specificity (Herrero et al., 2004; Lee et al., 2000). Domain III in the C-terminal region is responsible for the toxicity (de Maagd et al., 1996; Karlova et al., 2005), the formation of the ion channel and protection of the toxin from further proteolysis (Aronson and Shaib, 2001; Chandraa et al., 1999). Since a lectin-like fold exists in domain III of cry toxin, it will enhance toxicity because the carbohydrate motif can facilitate the toxin binding to receptors with specific glycodeterminants (Akao et al., 1999; Burton et al., 1999). Moreover, the binding of domain III with GalNAc will trigger the conformational change in the insertion domain (Li et al., 2001).

The information on the relationship between toxin structure and its function can be further used not only for toxin design to create new toxins for insect control (Crickmore et al., 2005; Saraswathy and Kumar, 2004), but also for the elucidation of the mode of action and the evolution of Bt resistance in insects.

1.2.2 Mode of Bt action

After ingestion by an insect, the Bt-toxin has to first pass through the peritrophic matrix (PM) diffusing into the midgut brush border, where it forms a membrane pore resulting in the death of the insect (Gill et al., 1992; Knowles and Dow, 1993; Knowles, 1994; Whalon and Wingerd, 2003). Since the insect midgut is the biological target site for cry toxins, the structure and function of the PM, midgut cell types, and Bt toxin receptors will be briefly reviewed.

The PM is a single semi-porous tube consisting of several layers composed of mucin-like glycoproteins, and chitin microfibrils (Lehane, 1997; Nation, 2002). The PM functions in the mechanical protection of the midgut brush border membrane (BBM), nutrient passage regulation, and compartmentation of the gut lumen (Lehane, 1997). It also serves as a barrier against the entry of virus, bacteria and bacterial products, such as the Bt protoxin (Hayakawa et al., 2004; Nation, 2002). Four types of midgut cells are found: columnar cells, goblet cells, regenerating stem cells and endocrine cells. Their functions are to secrete enzymes and to absorb nutrients via receptors on BBM of columnar cells. Since some adhesion and nutrient receptors are used by pathogens, the cellular and membrane composition of the gut lining is crucial for the action of Bt toxin.

The action of Bt toxin is characterised by a unique cascade of steps: proteolytic activation, complex formation that involves binding to receptors, pore formation and cell lysis (Knowles and Dow, 1993; Knowles, 1994; Whalon and Wingerd, 2003) (Fig. 1-2). After ingestion and digestion in the alkaline midgut by serine proteases, the protoxins of cry-endotoxins become soluble and activated. Activated Bt toxins first diffuse through the peritrophic matrix and reach the target receptor on the brush border membrane. The first important step is a reversible toxin-binding to receptors mediated by domains II and III (Ballester et al., 1999; Lee et al., 1999; Lee et al., 2000).

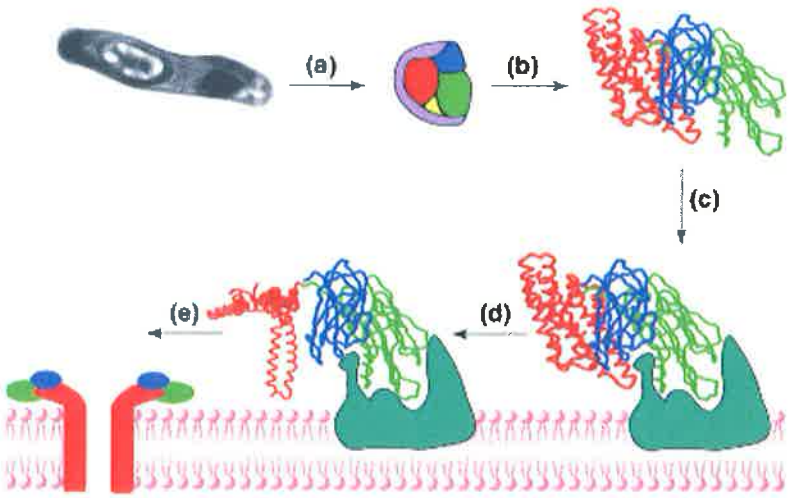

Fig. 1-2 Mode of action of Cry toxin. (a) Dissolved crystal in the gut juice. (b) Activation of toxin by gut proteases (c) Activated toxin binds to the receptors on the epithelical membrane, a process in which both domain II and domain III are involved. (d) Conformational change of domain I allowing a two-helix hairpin to insert into the membrane. (e) inserted toxins form pores probably as oligomers (de Maagd et al., 2001)

After binding to the receptors, domain I undergoes a conformational change to initiate the toxin insertion process (Knowles, 1994). The residue of Asn 135 in the helix α4 of domain I is involved in the toxin oligomerisation process (Rausell et al., 2004; Tigue et al., 2001), while the α5 helix plays an important role in toxin structure stabilisation and pore formation. The salt bridges in the domains I and II also affect the pore formation (Nunez-Valdez et al., 2001; Rausell et al., 2004) (Fig. 1-2).

After insertion into the membrane bilayer, the toxin interferes with the activity of K+/ amino acid transport, inhibiting K+ and amino acid assimilation in the gut lumen,

causing imbalance in pH, ion and other macromolecules in the gut, and finally leads to the lysis of the cell and insect death (English and Cantley, 1985; Knowles and Ellar, 1987; Leonardi et al., 1997; Reuveni and Dunn, 1991; Tran et al., 2001; Wolfersberger, 1991).

1.2.3 Bt application and Bt resistance

The first reported Bt resistance in an insect was from a stored grain pest *Plodia interpunctella* (Hubner), where several populations were continuously exposed to Cry1Ac crystals and spores for several generations (McGaughey, 1985). In the field, *Plutella xylostella* (L.) is still the only insect species that evolved resistance after exposure to Bt formulations (Ferre and Van Rie, 2002; Tabashnik et al., 2003). There is no direct evidence that any other insect species has evolved Bt resistance in Bt transgenic crops or with the application of Bt formulations. However, resistance has evolved in more than ten insect species selected under laboratory conditions (Ferre and Van Rie, 2002; Tabashnik et al., 1998), and in seven insect species exposed to transgenic crops in glasshouses (Tabashnik et al., 2003). The relative ease with which resistance has been obtained in containment suggests that insects have the potential to evolve Bt resistance in the field in the future (Tabashnik et al., 2003).

To effectively manage Bt resistance, the frequencies of resistance genes have been monitored in field populations (Gahan et al., 2001), and Bt resistance mechanisms extensively studied since the first cases of Bt resistance were identified from *P. interpunctella* (Ferre and Van Rie, 2002; McGaughey, 1985) (Table 1-1). Presently, several Bt resistance mechanisms are proposed based on laboratory studies (Ferre and Van Rie, 2002).

1.2.4 Inactivation of Bt toxin receptors

Although only one direct evidence showed that inactivation of receptors resulted in Bt resistance (Rajagopal et al., 2002), inactivation of Bt toxin receptors is the best-characterised Bt-resistance mechanism. Loss or reduction of toxin receptor activity resulting in Bt resistance in insects is designated as "Mode I" mechanism (Tabashnik et al., 1998). The binding of toxin to receptors can be divided into two steps: the first is a reversible and the second an irreversible step. It is the second and irreversible binding that determines the toxicity of the cry toxins (Liang et al., 1995). Furthermore, shared binding sites on the Bt toxin receptor for two different toxins will cause cross-resistance in insects (Granero et al., 1996).

Four types of Bt toxin receptors have been identified: aminopeptidase–N (APN), cadherin-like proteins, alkaline phosphatase (ALP), and glycolipid receptors (Table 1-2). APNs are frequently cited as receptors for Cry1Ac, but they are also receptors for Cry1Aa, and Cry1Ab. The cadherin-like receptor contains a number of cadherin repeats at the membrane-proximal regions, which contain the important toxin-binding site. The extracellular domain and a cytoplasmic domain in the BT-R1 receptor are involved in the signal transduction in the process of Bt toxicity (Dorsch et al., 2002; Gahan et al., 2001). The cadherin receptors are cited as receptors for Cry1Aa and Cry1Ab (Meng et al., 2001). Membrane-bound ALP is involved in phosphate transfer and assimilation, indicating that the ALP might affect the gut metabolism in insects, in addition to Bt binding at the gut lining (Jurat-Fuentes et al., 2002). Since ALP and APN are enriched in cholesterol-rich domains called lipid rafts it is possible that these membrane-structures are associated with Bt toxicity and resistance (Zhuang et al., 2002). In general, mutations causing Bt resistance through receptor inactivation are

recessive but the actual mode of inheritance is sometimes dependent on the toxin concentration. For example, Cry1Ac resistance in *Pectinophora gossypiella* (Saunders) was controlled by three resistance alleles on one locus. The resistance ratio can range between 300-3,000 fold. Furthermore, when the Bt toxin concentration is high, the inheritance of Bt resistance is mostly recessive, whereas when the toxin concentrations are in the medium range, the Bt resistance will be expressed as semi-dominant or partially recessive (Liu et al., 2001; Tabashnik et al., 2002). Similar results were found in *Chrysomela tremulae* (Fabricius) resistant to transgenic Bt (Cry3Aa) poplar trees (Augustin et al., 2004).

Given that resistance is recessive at high toxin concentrations and that rare mutations emerge initially in heterozygous insects, a strategy to delay the onset of resistance is based on the dilution of resistant alleles with susceptible alleles thus reducing the chance of two heterozygous insects mating and creating a homozygous resistant genotype. This resistance management strategy has been implemented in transgenic crops, which express the toxin at a relatively high level. In this approach, areas with transgenic crops are interspersed with non-transgenic crops, where susceptible insect populations exist and mate with rare mutant insects, thus keeping the heterozygous from mating with each other. This strategy is dependent on recessive modes of inheritance and random mating of susceptible and mutant insects. Recent observations of novel resistance mechanisms that are based on differnt mechanisms suggest dominant or semidominant traits and non-random mating, which may require novel resistance management strategies.

Table 1-2 List of the sequence characterised cry toxin receptors from insects

Insect	Receptor	Toxin	Author
M .sexta	APN2	Cry1Ab5	(Denolf et al., 1993)
M. sexta	APN 120kDa	Cry1Ac	(Knight et al., 1994)
M. sexta	APNs	Cry1Ac	(Knight et al., 1995)
Bombyx mori (L.)	APN 120&110 kDa	Cry1Aa	(Yaoi et al., 1997)
P. xylostella	APN1	Cry1A	
B. mori	APN 100 kDa	Cry1A	(Hua et al., 1998)
B. mori	APN 120kDa	Cry1Aa	(Yaoi et al., 1999)
H. virescens	APN 170&130kDa	Cry1Ac	(Oltean et al., 1999)
Lymantria dispar (L.)	APN1&2	Cry1Ac	(Garner et al., 1999)
E. postvittana	APN 120kDa	Cry1Ac&Cry1Ba	(Simpson and Newcomb, 2000)
P. xylostella	APN	Cry1A	(Zhu et al., 2000)
L. dispar	APN	Cry1A	
P. interpunctella	APN	Cry1A	
H. virescens	APN 110 kDa	Cry1Ac	(Banks et al., 2001)
H. armigera	APN	Cry1Ac	(Ingle et al., 2001)
B. mori	BmAPN3	Cry1Aa, Cry1Ab	(Nakanishi et al., 2002)
P. xylostella	PxAPN3	Cry1Aa, Cry1Ab	
H. armigera	APN1	Cry1Ac	(Rajagopal et al., 2003)
H. virescens	APN 110 kDa	Cry1Ac&Cry1Fa	(Banks et al., 2003)
M. sexta	Cadherin210kDa	Cry1Ab	(Vadlamudi et al., 1995)
B. mori	Cadherin	Cry1Aa	(Nagamatsu et al., 1999)
B. mori	BtR175b	Cry1Aa	(Ikawa et al., 2000)
L. dispar	Bt-R270	Cry1A	(Valaitis et al., 2001)
M. sexta	BT-R(1) (210-kDa)	Cry1A	(Midboe et al., 2003)
H. virescens	Cadherin	Cry1A	(Jurat Fuentes et al., 2004),
O. nubilalis	OnBt-R1,220kDa	Cry1Ab	(Flannagan et al., 2005)
P. xylostella	Lipid	Cry1Ac	(Kumaraswami et al., 2001)
H. virescens	Lipid rafts	Cry1Ac	(Darboux et al., 2002; Zhuang et al., 2002)
M. sexta	GPI-ALP,APN,actin	Cry1Ac	(McNall and Adang, 2003)
H. virescens	mALP	Cry1Ac	(Jurat-Fuentes and Adang, 2004)
C. elegans	Glycolipids	Cry5B	(Griffitts et al., 2005).
M. sexta	Glycolipid		

Bt-R, Cadherin; GPI-ALP; GPI anchored ALP; mALP, membrane-bound ALP

1.2.5 Unusual resistance mechanisms: Glycolipid receptors

Cry5B Bt-toxin, which shares 24% similarity to Cry1Ac, is toxic to nematodes. To understand the molecular mechanism and the genes involved in resistance, a mutational analysis was performed on *Caenorhabditis elegans* (Doughery). In this mutant screen, several mutant alleles (Bt-resistance, bre1-5), which allowed survival following exposure to the toxin, were isolated and shown to affect the modification of carbohydrate structures involved in Bt toxicity and the resistance mechanism (Griffitts et al., 2001). The implication is that different receptors can bind to the toxin through common carbohydrate structures and might cause cross-resistance to different toxins, which is a major threat to the usefulness of the Bt products in pest control (Griffitts et al., 2001). Changes in the carbohydrate structures are correlated with the loss of the BRE-5 function, which can lead to Bt resistance. Further studies found that the loss of three other glycosyltransferase genes bre-2, bre-3 and bre-4 also cause Cry5B resistance in *C. elegans* (Griffitts et al., 2003). Recently, it was found that alterations of Gly-T activity caused Bt resistance in nematodes via affecting glycolipids (Griffitts et al., 2005). This suggested that lipid and its associated proteins might play an important role in Bt resistance in invertebrates.

1.2.6 Inducible resistance mechanisms

Higher enzymatic activity may also lead to Bt-resistance by causing over-digestion of the mature toxins, which in turn may become non-toxic (Loseva et al., 2002). Since higher activity in specific gut proteases in resistant strains might be part of an immune reaction in insects, increased gut protease activity may be part of an induced Bt tolerance mechanism in insects. Immune components from the hemolymph, such as prophenoloxidase, are transported through the gut lining into the gut lumen (Fig. 1-3). Elicitor-mediated induction of gut secretions may be part of a general mechanism

involving the sequestration of toxins in the gut lumen. For example, increased activity of mannose phosphate isomerase (MPI) from resistant *P. xylostella* (Herrero et al., 2001), esterase from resistant *Helicoverpa armigera* (Hubner) (Gunning et al., 2005) and serine protease from *Choristoneura fumiferana* (Clemens) (Milne et al., 1998) bind the toxin and preclude the toxin from binding to receptors on the BBM. An unknown Ca-dependent enzyme from the midgut is also involved in the proteolytic cleavage of Bt-R1 (cadherin-like receptors) from *M. sexta*. The cleavage affects the adhesion activity and may serve as a protective function (Candas et al., 2002). This type of resistance mechanism would probably be inherited as dominant or semi-dominant alleles.

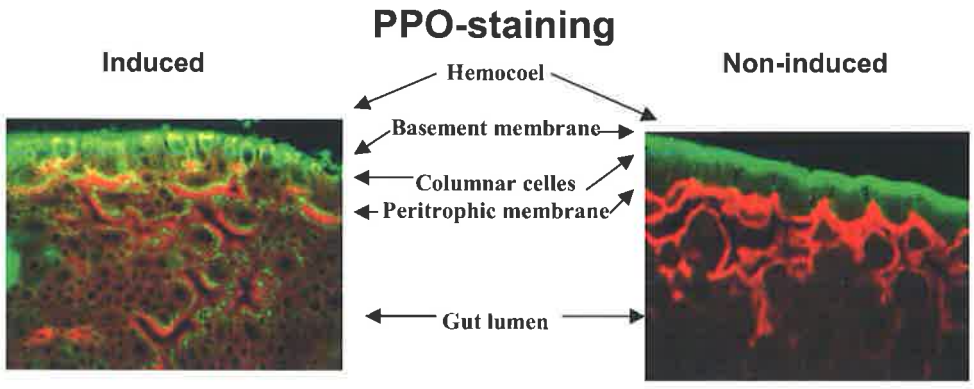

Fig. 1-3 Confocal microscopy of *P. xylostella* midgut before and after immune induction. Whole gut tissue was stained with anti-PO antibodies and counterstained with FITC-conjugated secondary antibodies. Tissues were inspected with indirect UV light for illuminating green and red fluorescing compounds. The yellow and green colours derived from anti-PO-stained antigens, which are visible in the basement membrane of the gut, in the space among gut cells and on the peritrophic membrane in the gut lumen. In lepidopteran insects the prophenoloxidase (PPO) is produced exclusively in hemocytes, which implies that PPO is transported from the hemolymph into the gut lumen through the gut cell lining. The peritrophic membrane is not labelled but shows autofluorescens in red-fluorescing UV light (Sarjan, 2002).

1.2.7 Bt (Cry1Ac) resistance in *H. armigera*

H. armigera is a major pest for cotton crops. Bt toxin genes (Cry1Ac) were engineered in cotton to control the pest. A Bt cotton variety INGARD®, which express the Cry1Ac toxin gene and originally targeted for *H. virescens*, was released to control *H. armigera* in Australia. Cry1Ac is 30 times less toxic to *H. armigera* than to *H. virescens* (Liao et al., 2002),. This combined with a decreased expression level of toxin during the development of the cotton (Liao et al., 2002), may have accelerated the development of Bt resistance in *H. armigera*.

H. armigera strains ANGR and ISOC4, which were selected in the laboratory from field populations, were used in this study. ANGR originated from crossing two field populations, AN02 and GR. ANGR is susceptible to the Bt toxin (Cry1Ac). ISOC4 was developed by crossing the BX strain, which was a mix of complex field-selected Cry1Ac-resistant populations consisting of BA2, BA8 and TO strains with ANGR (Akhurst et al., 2003). Four subsequent backcrosses with ANGR followed by selection with Bt produced a strain with more than 95% genetic homogeneity to ANGR. The resistance ratio for ISOC4 to ANGR is about 200 (Bird and Akhurst, 2004).

Akhurst et al (2003) found an unusual mode of Bt resistance in a Bt (Cry1Ac) resistant population, BX, of *H. armigera.* Bt resistance in the resistant strain was correlated with reduced binding to toxin receptors. Binding experiments showed that Cry1Ac only binds to one site in BBMV preparations from these insects when there is no detectable resistance. However, this major binding site was lost in selected strains once the resistance ratio reached 50 to 70 (Akhurst et al., 2003).

When BBMV preparations from the *H. armigera* strains were eluted from a Cry1Ac column, five proteins were bound to Cry1Ac: four of them were APNs and one was an unknown protein. The four APNs (which were assumed to be the Cry1Ac receptors) were sequenced and shown to be the same between resistant and susceptible larvae. The only difference between resistant and susceptible is the unknown protein, which was not identified due to the ambiguity of mass spectrometry data. This precluded the study of the biochemical basis of Bt resistance in this resistant strain (Angelucci et al., 2002).

Resistance was semi-dominant when the insects were subjected to high dose of Cry1Ac (Akhurst et al., 2003). Moreover, exposure to Bt toxin imposed a fitness cost, which is likely to derive from pleiotropic effects of the resistance gene rather than effects from other gene loci (Bird and Akhurst, 2004). This made the genetic basis of the resistant strain more complex.

Preliminary data suggest a complex resistance trait under low dose conditions, which could include dominant or semi-dominant effects caused by pleiotropic actions of the putative resistance gene. Another observation was that ISOC4 had an increased melanization reaction in the gut (Fig. 1-4). This suggested that resistance might not directly depend on the inactivation of one of the major receptor genes but on indirect pleiotropic effects from another unknown gene, which also affected developmental functions.

Fig. 1-4 Blackening of the peritrophic membrane and gut of resistant larvae. The grey appearance of the surface of epithelial cells in resistant larvae may be the result of melanization by hemolymph-derived phenoloxidase of components attached to the basement membrane. In addition black spots and dot-like structures were visible in the gut lumen and at the peritrophic membrane from resistant caterpillars only. Gut epithelial cells were not visibly different in the two strains (Sarjan, 2002).

1.2.8 Insect immune reactions

Insect immune reactions include cellular and humoral responses, which involve four major steps: recognition of nonself upstraem of signaling, followed by cell-free sequesteration and cellular signal transduction, which in turn is followed by downstream immune activation. The first step of an immune response is characterised by the recognition of pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), which can serve as initiators of prophenoxidase (PPO) and coagulation cascades. PPO cascdes involve the activation of a clip domain containing protease and the formation of melanin, which results in wound healing and

melanotic encapsulation of pathogens. Coagulation reactions are also important in wound healing and immune responses in insects (Li et al., 2002, Scherfer et al., 2004). Fibre (Hall et al., 1999) and globule formation (Schmidt & Theopold, 2004, Nardi et al., 2005,) are visible forms of coagulation reactions, which are affected by glycosylation reactions of pro-coagulants (Korayem et al., 2004).

Sugar modifications that cause coagulation in hemolymph plasma appear to be different in the gut lumen, where galactose (Gal)-specific lectins co-locate more strongly with toxin-binding globules (Sarjan, 2002), whereas GalNAc lectins are bound to the PM. In hemolymph, Gal-containing glycoproteins with pro-coagulant activities are mainly found inside hemocyte granules rather than in circulating plasma (Korayem et al., 2004, Scherfer et al., 2004). It is possible that glycoforms of pro-coagulants secreted into the gut lumen of immune-activated insects resemble pro-coagulants stored inside granules of hemocytes (Korayem et al., 2004). Interaction with sugar-binding proteins from pathogens and their toxins would thus form a first line of defence in the gut lumen. The assumption is that glycosylation changes affecting pro-coagulants to enhance sequestration of the toxin would be dominant, whereas glycosylation modifications that inactive Bt-receptors on the BBM are recessive.

One possible relationship between lectins and pro-coagulants has been predicted by a lectin-mediated endocytosis mechanism, where lectins interact with receptors to drive endocytosis but also with a soluble pro-coagulant to aggregate into coagulation products shaped as globules (Schmidt and Theopold, 2004). Lipophorin or other pro-coagulants are present in the gut lumen to shuttle lipids between the gut content and the brush border membrane. Since glycolipid is involved in Bt resistance (Griffitts et

al., 2005), Bt toxins could potentially interact with lipophorin or other pro-coagulants to form a coagulation reaction in the gut. Therefore the involvement of immune reactions in Bt resistance is a possibility that requires further experimental investigation.

The question is whether Bt resistance in *H. armigera* follows a dominant or semi-dominant mode of inheritance when subjected to low dosage of the Bt toxin? Since resistance in ISOC4 may not be directly associated with receptor inactivation, but involve changes in toxin-binding to BBMVs, this strain may have altered post-translational modifications of multiple proteins. For example, it is known that sugar modifications play an important role in immune reactions, which could affect pro-coagulants and modify receptors in the gut. Considering the glycosylation pathway conferring Bt resistance in *C. elegans* and its implication for insect Bt resistance, the modification of glycosylation pathways of Bt binding proteins might play an important role in Bt resistance in this *H. armigera* strain.

In addition to protein receptor modification, changes in pro-coagulants may affect tolerance to the toxin. Pro-coagulants such as lipophorin and hexamerin carry lipid molecules, which may be affected by altered glycosyltransferases. In this case, the changes may increase toxin-mediated coagulation reaction in the gut lumen causing sequestration of mature toxin before it can reach the gut lining.

1.3 Aims of the study

Bt toxin receptors are assumed to play an important role in Bt toxicity and alterations of receptor properties lead to the development of resistance in pests. Altered binding sites by post-translational modifications can also explain the development of Bt

resistance in pests. The loss of glycosyltransferase genes activity in nematodes, especially the novel hypothesis that the immune system might take part in the Bt resistance, may provide resistance mechanisms that require new strategies in the Bt resistance management. The question is what is the role of immune induction in Bt-tolerance.

The overall aims of the experiments were to find molecular differences between resistant and susceptible strains of *H. armigera* and to identify the molecular biology and the mode of inheritance of Bt resistance.

1. The first question is what is the Bt resistance mechanism in *H. armigera* under conditions where Bt toxin concentrations are low? The aim of the first set of experiments is to determine the Bt resistance mode of inheritance under low dosage of the toxin.

2. Since preliminary results showed that Bt resistant might be linked to melanisation in the gut, the role of Bt as an immune elicitor when applied in sublethal concentration was investigated. If a gut-derived immune induction is possible, it is conceivable that treated insects can produce immune inducible proteins. If they do, what is the molecular identity of the protein? Furthermore, the ultimate question is whether this protein is related to Bt resistance in *H. armigera*?

3. To better understand the importance of pro-coagulants in the gut lumen and insect immunity and further extend its possible role in Bt resistance, lipophorin protein domains involved in aggregation and an induced pro-

coagulant protein were sequenced, expressed, and their functions in immune reactions were further studied in this project.

4. If resistance is based on an inducible mechanism, a prediction is that it is not visible in the next generation, unless some of the induced activity is transmitted through the germ line by a paternal or maternal effect. Since immune related pathways exist in oogenesis and embryogenesis, the most likely mode of transmission is a maternal effect. Whether immune-induced components are incorporated into the oocyte and whether any of the factors are related to insect Bt resistance, and transferred through the germ line by a maternal effect will be examined.

Chapter 2 Materials and Methods

2.1 Insects

2.1.1 *H. armigera*

Resistant ISOC4 and susceptible ANGR strains of *H. armigera* were used in the project. These have been described in detail by (Akhurst et al., 2003) and Bird and Akhurst (Bird and Akhurst, 2004).

2.1.2 *Galleria mellonella*

G. mellonella was used for lipophorin studies. Larvae were reared on artificial diet (Appendix 3.5) at 25°C with a 10/14 h light/dark cycle (Li et al., 2002).

2.2 Bt toxin production, purification, activation, and concentration

To test the difference between ANGR and ISOC4 BBMVs with regard to the binding to Cry1Ac toxin, the protoxin was prepared and activated as described by (Liao et al., 2002). The prepared protoxin is shown in Fig. 2-1 of this chapter, and the activated Cry1Ac in Fig. 2-2.

2.2.1 Bt preparation and purification from HD73 strain

The HD73 strain contains a plasmid that produces Cry1Ac as the only cry endotoxin. Cry1Ac Bt pro-toxin was prepared as described by (Liao et al., 2002). The method of (Luthe, 1983) was used for making up 60%, 70%, 80%, and 90% (w/v) sucrose solutions respectively which were used in the purification step. The protoxin was used for Bt bioassays, and proteolytic activation of mature toxin.

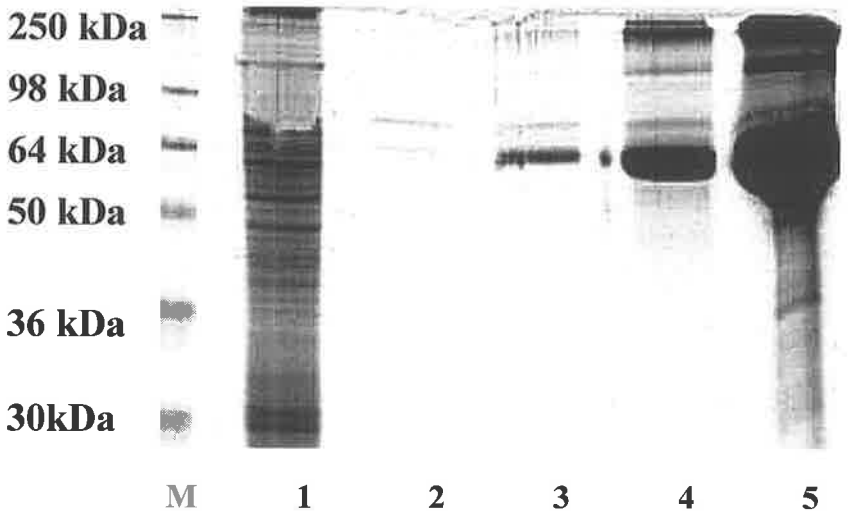

Fig. 2-1 Pro-toxin concentrations were determined by comparing with known concentrations of BSA. 20 µl of sample were loaded on each lane under reducing conditions on 10% SDS-PAGE. Lane 1 is the protoxin, Lane 2 to 5 were BSA solutions with known concentrations in the following order: 0.001, 0.01, 0.1, and 1 µg/µl. This batch of protoxin concentration is estimated to range between 0.001 and 0.01 µg/µl based on this method. Star indicates the 135-kDa Cry1Ac protoxin and arrow shows the BSA size

2.2.2 δ-endotoxin crystal solubilization and activation

The method proposed by (Burton et al., 1999) was used for solubilisation and activation of the δ-endotoxin crystal. The active toxin was used for toxin-binding and relevant experiments.

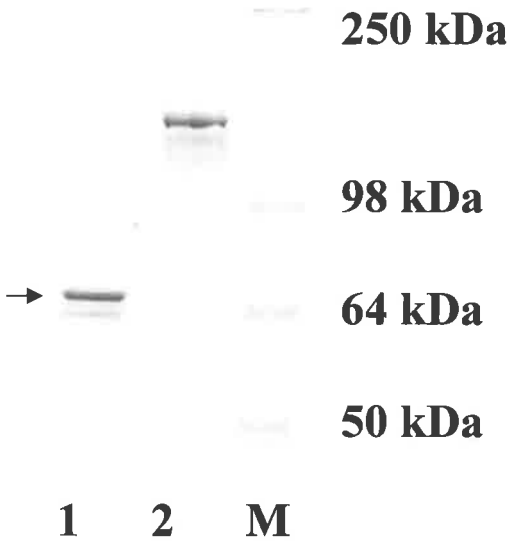

Fig. 2-2 Coomassie staining of the purified Cry1Ac protoxin and activated toxin Separated on a SDS-PAGE (10%) after heating at 100°C. 1: trypsin digested Cry1Ac; 2: Cry1Ac pro-toxin; M: SeeBlue® Marker. Arrow indicated the activated Cry1Ac toxin at 69 kDa. Below is a minor band of over digested toxin.

2.3 Bt bioassays

2.3.1 Genetic crosses

2.3.1.1 Preparation of *H. armigera* diet and rearing conditions

See Appendix 3.5

2.3.1.2 Crosses

To uncover possible genetic contribution of immune-induction in *H. armigera,* after the insect was subjected to low dosage of Bt toxin (Cry1Ac), and to examine how immune-related Bt-tolerance mechanisms are transmitted to the next generation, four

crosses were performed, susceptible **x** susceptible (SxS), resistant x resistant (RxR), susceptible female x resistant male (SxR) and resistant female x susceptible male (RxS).

Based on the available information about the ranges of dosages used for Bt bioassays, a preliminary experiment was set up to determine the optimal toxin concentration to separate the two populations. Preliminary results showed that 1µg/ml of Cry1Ac/spores from the HD73 strain was suitable for distinction between resistant and susceptible larvae (results not shown). Since toxin dosages in the present experiment were relatively low and the bioassay period relatively long, the diet incorporation method was used for this method, which is equivalent to the toxin expression level of later stage transgenic crops (Liao et al., 2002) but may differ with regard to the actual active concentration of the toxin. For each of the crosses, similar weight, four-day-old larvae from 20 pairs of adults were exposed to a spore/Cry1Ac preparation of HD73 incorporated into the *H. armigera* diet, at a concentration of 1µg/ml as determined by in-gel comparison with a BSA standard solution. The protoxin was shown to be 1 to 10 ng as indicated in Fig. 2-1.

24-well plates, containing about 1 ml diet mixed with and without Bt toxin were used (Liao et al., 2002). The wells were covered with plastic balls, which allowed aeration but no escape for larvae. Each well contained one larva at the start the bioassay. Numbers of larvae used for each cross were: SxS n=144, RxR n=144, SxR n=192, RxS n=120. Mortality was recorded daily for seven days, at which point all susceptible insects had died.

2.3.1.3 Statistical analysis

The data were analysed by Cox's proportional hazard model, using Efron's partial likelihood method (Efron, 1977). To determine the differences between the sexes, the SxS cross was treated as the baseline hazard and the resistant males and resistant females as separate covariates, assumed to be acting multiplicatively. To determine the differences between the crosses, the SxS cross was treated as the baseline hazard and the other three crosses as separate covariates.

2.4 Induction of the immune response

2.4.1 Immune-induction

The induction of melanization and other defence reactions was carried out by exposing late 4^{th} to early 5^{th} instar larvae from the susceptible strain to a sublethal dose of Cry1Ac protoxin (1 μg per ml of artificial diet) for various time periods as indicated. Note: that this concentration is lethal for younger larvae but not for larger larvae (see 2.3.1.2).

2.4.2 Lectin-mediated aggregation

To test wether any immune induced proteins could interact with Cry1Ac, Bt (Cry1Ac) induced hemolymph plasma was incubated with active Cry1Ac for different time periods. Actived Cry1Ac toxin (2 μg) was incubated with the 20μg of hemolymph protein (from 1h Cry1Ac-induced $ISOC_4$) for 10, 20 and 30 min at RT, then centrifuged for 1 min at 12,000 x g. Attempts to separate the pellelt using chaotropic substances such as guanidine chloride and SDS were not successful, therefore, the supernatant was separated by 12% SDS-PAGE, and any protein missing from the protein pattern was used as an indication that it is involved in aggregation

reactions. Hemolymph plasma protein (20 μg) from non-induced larvae was used as a negative control.

2.5 Protein extraction, concentration, separation, and identification

2.5.1 Protein extraction

2.5.1.1 Hemolymph, gut, fat body, and egg preparations

Hemolymph, which was used for immune induction with either Cry1Ac or LPS, was collected from early 5th instars by the method of (Trowell et al., 2000).(2000) (2000) No PTU was added to the hemolymph, when it was used for melanization measurements. Gut, fat body, and egg preparations were performed according to (Trowell et al., 2000) and were used for either protein (for SDS-PAGE, Western blot, and HPLC) or RNA (for RNAi, RT-PCR, or 5'RACE study) extraction.

2.5.1.2 BBMV preparation

BBMV were prepared based on the method of (Wolfersberger et al., 1987). In brief, the midgut was cut and its content was rinsed with buffer A (300 mM mannitol, 5mM EGTA, 17 mM Tris-HCl, pH7.5), and weighed. Then the gut was homogenised in buffer A and 24 mM $MgCl_2$, followed by two centrifugation steps to enrich the BBMVs, which were suspended in Buffer A for relevant protein analysis.

2.5.2 Protein concentration estimates

The concentration of the activated toxin and the insect proteins of interest were determined by the method of (Bradford, 1976). The concentration of the protoxin was determined by the SDS-PAGE method, in which a set of BSA solutions with known

concentration were used as standard, with the same volume of protoxin was loaded on the gel along with the BSA samples. After staining and destaining of the SDS-PAGE gel, the protoxin concentration was determined visually by comparing with the standard BSA samples (Fig. 2-1).

2.5.3 Protein separation

In order to characterize the proteins of interest (immune induced protein, alkaline phosphatase), proteins were separated based on their molecular weight, hydrophobicity, and affinity to ligands.

2.5.3.1 SDS-PAGE and Western blots

One-dimensional SDS-PAGE (Laemmli, 1970) was regularly used in the project. The molecular weights of proteins were estimated based on the relative migration rate compared with molecular markers. After gel electrophoresis, the proteins were transferred to nitrocellulose membrane (Western blot) in the Towbin transfer buffer (Appendix 3.1) for protein identification by direct N-terminal sequencing or antibody detection (Towbin et al., 1979).

2.5.3.2 High performance liquid chromatography (HPLC)

The proteins of interest were loaded on a Prous 20R1 200 x 2.1 mm column and eluted at the flow rate of 0.3 ml/min using a gradient of 5 to 100% of "Solvent B" [0.04% trifluoroacetic acid (TFA) in 70% acetonitril] against "Solvent A" (0.05% TFA in water) over 60 minutes (Table 2-1). The HPLC was run in a gradient as indicated in Table 2-2. Fractions were detected by absorbance at 214 nm and protein fractions were collected manually.

2.5.3.4 Affinity chromatography

This is an approach for purifying recombinant His-containing proteins according to their affinity difference to certain resins, which may be conjugated with Ni ions. Proteins with His residues or particular binding properties will be specifically absorbed and eluted from the column. Purification of several recombinant proteins of interest (such as vWD, hemocyanin C and hemocyanin M domains) were eluted from Ni-NTA resin (GIBCOBRL®) using 6 M Guanidine hydrochloride according to the manufactureis protocol.

Table 2-1 The parameters for HPLC

Stop time	60 min
Flow rate	0.3 ml/min
Oven temperature	40°C
Peak width	0.04 min
Sampling interval	0.16 second
Wave length	214 /280 nm

Table 2-2 HPLC Gradient

Time (min)	% Solvent A	% Solvent B
0	95	5
40	30	70
45	0	100
50	0	100
51	95	5

2.5.4 Protein detection

Proteins of interest were detected by either staining polyacrylamide gels with Coomassie Brilliant Blue R-250 stain or staining Western blots with dye, lectin or conjugated secondary antibody (Merril and Washart, 1988).

2.5.4.1 Polyacrylamide gel staining

To visualize the proteins from gels, the gels were stained with either Coomassie Brilliant Blue R-250 stain, or with zinc salt. After protein separation according to 2.5.3, the protein was stained with Coomassie Brilliant Blue R-250 stain in methanol and acetic acid solution (Appendix 3.1) for 10 min, then destained in methanol and acetic acid solution (Appendix 3.1) for 1 hour or overnight (Merril and Washart, 1988). The negative stain was performed based on the method of (Hardy, 2001). In brief, after separation by SDS-PAGE, the gel was washed with water for 15 min. with 3 changes, then, soaked in 200 mM imidazole for 10 min. The gel was soaked in 200 mM $ZnSO_4$ or $Zn(AcO)_2$ for 1 to 5 min and visualised using a light box.

2.5.4.2 Western blot

Proteins immobilized on membrane according to 5.3.1.were used either for normal protein detection or for the subsequent direct N-terminal sequencing. For N-terminal peptide sequencing, the staining on the blot was performed according to the method provided by the manufacture (Polyvinylidene diflouride or PVDF, Hybond™-P, Amersham pharmacia biotech).

2.5.4.3 Glycoprotein identification

Glycoproteins, which have different oligosaccharides, could be detected by staining with available peroxidase-labelled lectins on Western blots. The lectins were used in the current project is listed in Table 2-3. After immobilisation onto a nitrocellulose membrane, the blot was first blocked in TBST+0.1-3% BSA solution for one hour, then, incubated with lectin at 1:10000 for overnight or 1:5000 for two hours. The blot was washed extensively with TBST for one hour, changing the solution with four times, followed by peroxidase reaction developing buffer (Appendix 3.1).

Table 2-3 List of lectins used in the current project

Lectin	Common name	Sugar specificity
Arachis hypogaea (peanut)	PNA	Galactose-specific
Canavalia ensiformis (Jack bean)	Con A	Non-reducing α-D-glucose and α-D-mannose
Dolichos biflorus	DBA	N-acetylgalactosamine (GalNAc)
Erythrina Cristagalli	ECL(ECA)	Galactose & galactosyl (b -1,4) N-acetylglucosamine.
Glycine max (Soybean)	SBA	N-acetylgalactosamine (GalNAc)-specific lectins
Helix pomatia	HPL	alpha-N-acetylgalactosamine residues
Jacalin		Galactosyl (β-1,3) N-acetylgalactosamine (O-link)
Triticum vulgaris (wheat germ)	WBA	N-acetylglucosamine, Sialic acid
Bandeiraea simplicifolia	BS-1	GalNAc
Vicia vilosa	VVL	GalNAc

2.5.4.4 Immunological detection

After proteins were attached to the blot, the membrane was first blocked in Blocking Buffer I (Appendix 3.1) for one hour to occupy the unspecific binding sites, followed by incubation with a specific antibody for the protein of interest in the same buffer for a time period (normally overnight at 1:5000 ratio). The next day, the blot was washed with 1X TBST for one hour with four changes of washing buffer. The blot was then incubated with the enzyme-conjugated (normally alkaline phosphatase or peroxidase) secondary antibody for one hour in the Blocking Buffer II (Appendix 3.1), and with the same washing buffer of 1X TBST. The proteins were visualised either by a colour reaction in colour developing buffers or by a luminofluorescence in light reaction buffer. The light reaction was visualised on film. The antibodies used in the project are shown in Appendix 4.

2.5.4.5 Protein detection by luminofluorescence

Once the proteins were immobilised on nitrocellulose membrane, the proteins were visualised by a chemical-light reaction in the presence of H_2O_2. The pre-requirement for this detection method is that the blot should be stained with peroxidase conjugated antibodies or lectins (Zhang, QS Per. Comm.). In brief, after final block with BSA and extensively wash with 1XTBST, the blot was incubated with a chemical mix of 100 µl of p-coumaric acid stock solution and 10 ml of luminal- H_2O_2 stock solution for ca. 1 min at RT. Then, the X-ray film was exposed for certain time period on top of the blot, which was sealed in a plastic wrap, followed by development in the Curix 60 (AGFA, CP1000) X-ray film developer.

2.5.4.6 ALP detection by the zymogram method

To test whether elevated gut protease is related Bt resistance in *H. armigera*, zymogram was originally used for enzymatic analysis, but in the current project, the ALP protein was detected by chance via this method. The zymogram method was essentially performed based on the method of (Garciacarreno F. L. et al., 1993). In brief, the BBMV preparations were heated at 37˚C in non-reduced loading buffer, then separated on 12% SDS-PAGE at 4˚C. Set the voltage at 50 V. Stained with CBBR-250 in acetic and methanol solution, and destained in acetic and methanol solution.

2.5.5 Protein sequence analysis

Protein sequences were determined by the following two approaches.

2.5.5.1 N-terminal sequence

After staining with methanol-based Coomassie Blue as described in section 5.4.2, the bands of interested were cut from the Western blot, destained with methanol, and washed with water. The filter piece was sent to the Australia Proteome Analysis Facility in Maquarie University (APAF), Sydney for N-terminal sequencing. Automated Edman degradation was also carried out using an Applied Biosystems 494 Procise Sequencing System (Edman and Begg, 1967).

2.5.5.2 Internal sequence

Since most of the biologically important extracellular proteins of interest were blocked at the N-terminal residue, it is therefore difficult to get an N-terminal sequence (Simpson and Reid, 1998). To characterise a protein sequence, the protein

was either digested in-gel or on-membrane to obtain peptides from the internal protein sequence.

The in-gel digestion was performed based on the method of (Simpson and Reid, 1998). In brief, the protein band was cut from gel and cut into small pieces, then destained in destain buffer (100mM NH_4HCO_3), followed by reduction in 3mM β-mercaptoethanol and alkylation in 3 mM iodoacetamide followed by drying under cool conditions. The samples were then digested with trypsin O/N, followed by extraction with TFA. The extracts contain the polypeptides and were separated in RP-HPLC, and further sequenced. Peptide sequences were used for protein-protein blast with the NCBI gene bank to find the homologues. On-membrane chemical dgestion with CNBr was based on the method of (van Montfort et al., 2002).

2.5.5.2.1 Reverse Phase-HPLC

After digestion, HPLC was performed to separate the digested peptides as that of 2.5.3.2. The column used for peptide separation was a VYDAC® reverse phase C18 column. The parameters for RP-HPLC and the gradient for RP-HPLC were those described in the Table 2-4 and 2-5 respectively. A blank using a similar sized gel sample from the SDS-PAGE was included in the trypsin digestion as a negative control. The negative control provided a baseline for comparison of RP-HPLC results.

2.5.5.2.2 Peptide sequencing

Peptide peaks from the RP-HPLC (approximately 100 pico-mol) were vacuum-dried, reconstituted in 8M urea, containing 0.1M NH_4HCO_3 and 4mM DL-dithiotreitol

(DTT) and finally alkylated by addition of sodium iodoacetate to a final concentration of 10mM. The samples were acidified with TFA to pH 3 to stop the reaction. Peptide sequencing was carried out using a Hewlet Packard G1000A Protein Sequencer (Edman and Begg, 1967) by the Biotechnology Unit at Waite Campus, The University of Adelaide. The peptide sequences are listed in Table 2-6.

2.5.5.2.3 Peptide sequence determined by MALDI-TOF

MALDI-TOF, or Matrix-assited laser desorption ionization-time of flight, was used to characterize peptides. It involved three steps. The first step was the ionisation of protein molecules in MALDI. The protein molecules were embedded into a matrix of selected ultraviolet (UV) absorbing organic salts and bombarding with a UV laser beam. After absorption, the UV light led to a local, explosive evaporation of the matrix material. The resulting peptide molecules were pulled into the vacuum system of the mass spectrometer. The second step was to mass through "Time-of-flight Mass Spectrometer" by using electric and magnetic fields to separate ions. Ion separation is dependent on the mass-to-charge value. The last step is the protein identification by comparison with the available database.

The protein of interest (an 85 kDa immune-induced protein) was digested with trypsin, under standardised conditions and the resulting peptides desalted into the QTOF2 mass spectrometer through a C18 reverse phase silica column at the Hanson Protein Core Facility, Division of Human Immunology, I.M.V.S., South Australia. Automated data directed analysis was carried out, to attempt to determine sequence tags from the tryptic fragments from the digestion. The recorded spectra were calibrated against a standard solution of [Glu1]-Fibrinopeptide B. The data are

searched against the NCBI Non-Redundant database (18 June 2004) without species restriction.

Table 2-4 The parameters for RP-HPLC

Stop time	78 min
Post time	5 min
Oven temperature	40°C
Peak width	0.04 min
Sampling interval	0.16 second
Wave length	214 /280 nm

Table 2-5 RP-HPLC gradient

Time (min)	% Solvent A	% Solvent B
0	95	5
60	30	70
65	0	100
68	0	100
69	95	5

2.5.5.3 Protein-protein BLAST

The peptide or combined peptide sequences from the 85 kDa protein were used for search for short, nearly exact matches with the available protein sequence database from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Identities of the

proteins of interest were determined by using information from both protein-protein blast and antibody detection.

Table 2-6 List of peptide sequences

No	peptides	Protein
26.	LTDEPFGFPVNRPL	Hexamerin
37	NIEHYXXVVAVXTY	Hexamerin
14	H/L T/A P/E E/H IEVPH	Hexamerin
14	DXPDIAHQLVHHHPG	Alkaline phosphatase
34	IDHAHHDNLVXLALD	Alkaline phosphatase
17-1	AFLPNTVVDDMGSYG	Alkaline phosphatase
17-2	TWENDGEQSQE	Alkaline phosphatase

2.6 RNA extraction and sequence analysis

2.6.1 RNA isolation

To isolate the coding DNA fragments and to study the expression of genes of interest, RNAs from various tissues were isolated with a one-step method. This one-step method was adapted from (Chomczynski and Sacchi, 1987).

2.6.2 RNA concentration determination

The concentration of RNA was determined by measuring the absorbance at 260 nm where an absorbance of 1 is equivalent to 40 μg ml^{-1}. The purity of RNA was tested by calculating the ratio of spectrophotometry readings at 260 nm and 280 nm (A_{260}/A_{280}). Pure RNA has a ratio of 2.

2.6.3 DNA primer design

To further characterise the proteins, peptide sequence information (Table 2-6) and the codon usage of *H. armigera* available online (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=Helicoverpa+armigera+[gbinv) were used for the development of degenerate primers. Primers were synthesized by Proligo® or Geneworks® and listed are listed in Table 2-7 to Table 2-10.

2.6.4 Sequences strategies for genes of interest

2.6.4.1 RT-PCR, 5'RACE, and 3'RACE

2.6.4.1.1 RT-PCR (Reverse Transcript-PCR)

To amplify the genes of interest by degenerate primers, which were designed based on amino acid peptide sequences, and also for studying the gene expression, RT-PCR was performed according to the method of (Wu et al., 1997). This method includes three major steps: RNA denaturation, RNA reverse transcription (RT) into DNA, and amplification of the cDNA with the degenerate primers.

Table 2-7 Primers for amplification of apolipophorin domains from *G. mellonella*

No.	Primer name	Primer sequences
1	GmLPF1	GCGAGCCACTTCCTTCC
2	GmLPR1	ACCGTTTATCACTGGCC
3	GmLIPF2	GCCGGCGATGTGCTTGC
4	GmLIPR2	CGTGAAGATGTGTTGTCC
5	GmLPF3	CGCTCTTCAGTTTCAAGTC
6	GmLPF4	GCAAAGTATGAATTGGG
7	GmLPF5	GGCGTACTGCTCTGCTAAACTC
8	GmLPF6	CTTCTCACAGTTGGTCTTTATG

Table 2-8 Degenerate primers for amplification of the vWD domain from *H. armigera* and *P. xylostella*

InsectvWD-1-F:	CACATCTTCACGTTCGACG
InsectvWD-1-R:	CGAAGTAGCACACC

Table 2-9 Degenerate primers for the amplification of hexamerin coding DNA

No.	Primer name	primer sequence
1	HaAngr26F	GACGARCCNTTYGGNTTC
2	HalspGSP$_3$/R	TGACTGGGAAACCGAATG
3	AUAP	GGCCACGCGTCGACTAGTAC
4	HalspGSP$_4$/R	TGTCAATGGAATCACGAG
5	UAP	CUACUACUACUACGCCACGCGTCGACTAGTAC
6	HalspGSP$_5$/R	CGCCAAGAAGAACTTGAC
7	Hahex5F	TAYATGGARAGAYTGTC
8	Hahex5f-1	ATGGSTCKWTTGRKBTTGT
9	HalspGSP$_6$	AGACAGTCTCTCCATGTA

Table 2-10 Degenerate primers for the amplification of alkaline phosphatase coding DNA

No.	Primer name	Primer sequence
1	HaALPF1	AGACAGTCTCTCCATGTA

2.6.4.1.2 RACE (Rapid Amplification of cDNA Ends)

RACE is a method for amplification of nucleic acid sequences from a mRNA template between a defined internal site and unknown sequences at either the 3' or the 5'- end of the mRNA (Frohman et al., 1988). Since the natural poly (A) tail in mRNA was used as a generic priming site for PCR amplification, the 3'-RACE was performed by using a gene specific primer and oligo-dT adapter primer. Therefore, the unknown 3'mRNA sequence that lies between the exon and the poly (A) tail could be determined. In addition, 5'RACE was used to capture the isolation and determination of 5'-ends from low copy messenger-RNA. As in the normal RT-PCR, the first strand cDNA synthesis was primed using a gene-specific antisense oligonucleotide (GSP). RNA template was removed by RNAse mix. The 1^{st}-strand product was purified followed by homopolymeric-tailing of cDNA. Finally, the target cDNA was amplified by one or two rounds of PCRs.

2.6.4.2 Gel electrophoresis of DNA, and DNA extraction from gel

DNA was separated by agarose-gel electrophoresis. It was based on the method of (Sambrook et al., 1989a). The DNAs on the agarose gel were extracted and purified by an Eppendorf kit. The gel was heat at 50°C for 10 min in extraction solution. The extracted DNA was decant into a column and eluted with MQ water.

2.6.4.3 Ligation and transformation of genes to pGEM-T easy vectors

The purified DNA was further ligated to the sequence vector pGEM-T easy vector, and transformation of *JM109* with the ligation mix performed at 37°C overnight according to the manufacturer's protocol. After transformation, the gene was first

screened by PCR with M13 forward and reverse primers. Then, after extraction with Qiagen kit, the plasmid was extracted in three different buffers, followed by centrifugation at full speed for 10 min. The supernatant was decanted into a column and elute with water. Plasmid DNA was digested with *EcoR I* to further confirms the PCR result.

2.6.4.4 Preparation of competent cells of M15 and JM109 for transformation

Preparation of competent cells of M15 and JM109 were performed as described by the method of (Sambrook et al., 1989b).

2.6.4.5 Sequencing of PCR fragments and purification

Once the correct insert was determined, the plasmid DNA was then used as template for subsequent DNA sequencing analysis. The procedures for sequencing PCR fragments, PCR programs, and recovery of PCR products were provided by the DNA sequence centre of IMVS, South Australia. The original DNA sequence was analysed with "SeqEd v1.0.3" or "Editseq" (Lasergene product). The gene sequence was further extended until the full-length was determined by using internal primers chosen by the ANGIS program.

2.6.4.6 Partial sequence of apolipophorin I from *G. mellonella*

Two clones (No. 2 and 3), which contained the two domains of apolipophorin from *G. mellonella* were used as templates (Li et al., 2002). A 4526-bp partial sequence (see Appendix 5) was produced after 10 rounds of PCR amplification according to the following strategy (Fig. 2-3).

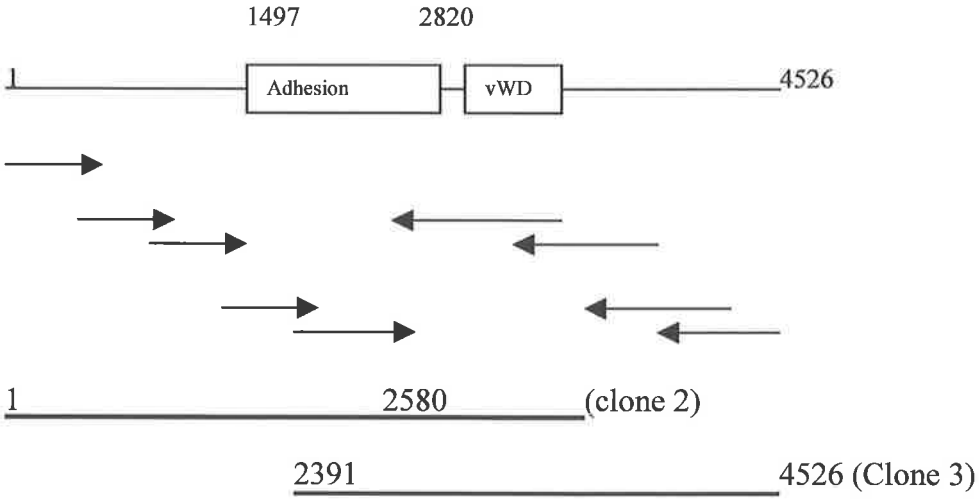

Fig. 2-3 Strategy for sequencing of the apolipophorin I from *G. mellonella*

2.6.4.7 VWD sequence from *H. armigera* and *P. xylostella*

The vWD sequences from *H. armigera* and *P. xylostella* were amplified using the standard RT-PCR approach. Degenerate primers were obtained from alignment of five available insect vWD domains of apolipophorin. The nucleotide and deduced protein sequences were then determined by the methods described in section 2.6.4.1 to 2.6.4.4 of this chapter. The sequences were in the Appendix 5.

2.6.4.8 Strategy for sequencing hexamerin cDNA from *H. armigera*

The strategy for sequencing hexamerin cDNA is shown in Fig. 2-4.

2.6.5 Blast, alignment, and characterisation

2.6.5.1 Blastp analysis of hexamerin

The amino acid sequences were deduced from the longest available open reading frame (ORF). Conserved motifs were identified through comparisons with known proteins in the Genebank by using Blastp from NCBI.

2.6.5.2 Megalign of proteins

Deduced protein sequences were alignded with other highly similar sequences from different organisms by using the CLUSTAL method through "Genedoc" and "Megalign" (Lasergene product) programs.

2.6.5.3 Structural and function prediction

Theoretical isoelectric points, molecular weights and charges of proteins of interest were predicted using "Editseq" software. All other structural parameter analyses for the proteins of interest were accessed through the ExPASy molecular biology server (http://us.expasy.org/tools). Signal peptides of proteins of interest were determined by the method of (Bendtsen et al., 2004). N-Glycosylation sites of the deduced amino acid sequences were based on NetGlyc 1.0. (http://www.cbs.dtu.dk/services/NetNGlyc/). O-Glycosylation sites of the deduced amino acid sequences were based on YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/). Phosphorylation sites were prediction in the deduced amino acid sequences by using NetPhos 2.0. (http://www.cbs.dtu.dk/services/NetPhos/). Prediction of Coiled-coil regions of hexamerin was performed by Coils software (http://www.ch.embnet.org/software/COILS-form.html). The NCBI gene bank accession number for these sequences are listed in Appendix 5

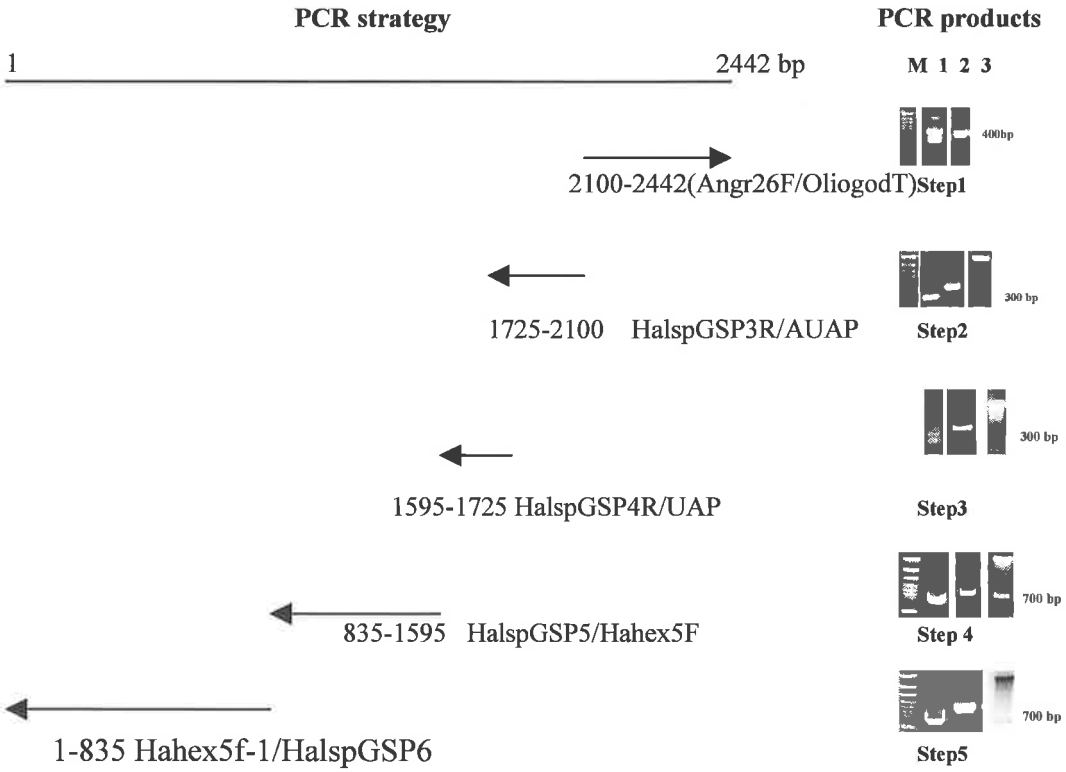

Fig. 2-4 Detailed strategies for cloning of hexamerin from *H. armigera*

The left side shows the products from each step. M, Marker; 1, Lane 1, PCR product; 2, PCR product ligated into pGEM-T easy vector; 3, vector containing the correct insert was digested with EcoR I to further confirm the correct ligation.

Step1. 3'RACE of hexamerin from *H. armigera*. A degenerate primer HaAngr26F (see sequences in Table 2-9), which was obtained from peptide 26, and an oligo dT primer were used. The 3'RACE was performed in a PCR program of 95°C, 4 min, 85°C hold, 94°C, 30 s, 45°C 30 s72°C60s; 94°C 30s, 50°C, 30s, 72°C, 60s 34 cycles. A 400 bp cDNA was obtained after 3'RACE. The cDNA was further ligated in pGEM-Teasy vector and sequenced. (1) 400 bp product (2) 400 bp in pGEM-T easy vector.

Step 2. Three Gene Specific Primers (GSP1, 2, and 3, sequence in Table 2-9) were designed based on the 3'RACE result for step 1. AUAP and HalspGSP/R primers were used for the first 5'RACE cycle. The PCR program was 94°C, 1', 94°C, 30'', 55°C, 30'', 72°C, 1'45'', 35 cycles. A 300 bp product was obtained and sequenced

Step3. A further 5'RACE was performed by using HalspGSP/R4, which was designed according the results from step 2, and UAP. A 300-bp cDNA in size was obtained and sequenced.

Step 4. The first degenerated RT-PCR was initiated by using a degenerate primer Hahex5end-F (sequence in Table 2-9), which was designed based on the alignment information, and GSP5 (designed from step 2). Its product length was 700-bp in size and sequenced.

Step 5. The second degenerate RT-PCR was initiated by using a degenerate primer Hahex5F-1 (sequence in Table 2-9), which was designed based on the alignment information, and HalspGSP6 (designed from step 4). Its product length was 900-bp in size and sequenced.

2.7 Protein expression and purification

To study the functions of the genes of interest, the sequenced genes were expressed in the pQE30 or pQE31 (Qiagen®) bacterial expression vector systems depending on the open reading frame of the coding fragment. One pQE31 (for the vWD domain from *G. mellonella*) and two pQE30 (for the two domains of hexamerin from *H. armigera*) expression vectors were constructed.

2.7.1 Primers and vector construction

Primers were designed with restriction sites flanking both ends to facilitate the expression-vector construction. Primers were listed in Table 2-11. The PCRs were performed with a reaction mixture of 0.2 μg of plasmid as template, 5 μl of 10x PCR reaction buffer, 3 μl of 25 mM $MgCl_2$, 1 μl of 15 mM dNTPmix, 2 μl of each primer, 0.5 μl of Taq polymerase and 36.5 μl of MQ water. After pre-denaturing the PCR reaction mixture at 94°C for 2 min, the PCRs were performed for 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72°C for 1 min, followed by 5 min extension at 72°C. The PCR products of interest were first ligated into the pGEM-T easy vector to facilitate further ligation into the expression pQE30 or pQE31 vector. The amplified DNAs were digested with restriction enzymes in buffer E at 37°C overnight and ligated into the correponding (Qiaexpress®) expression vectors, which were pre-cut with the same restriction enzymes, followed by gel extraction with an Eppendorf kit. The DNAs were further purified using ethanol precipitation. The inserts were ligated into the vectors and transformed to M_{15} overnight at 37°C. The correct inserts were selected by PCR (primers are in Table 2-12) and enzyme digestion, and further confirmed by DNA sequencing.

Table 2-11 Primers used for vector constructions

Protein	Primer name	Primers	Insect
VWD domain	GmvWDbamH1F:	CGCGGGATCCATTCGATGAAATTCCTGC	*G. mellonella*
VWD domain	GmvWDHindIII-R:	GCGCAAGCTTTCCATTGGGCAGTCTGAAG	*G. mellonella*
Adhesion	GmVaaBamHI-F	CGCGGGATCCGTAAAGGAGAACTTGCAG	*G .mellonella*
Adhesion	GmVaaHindIII-R	GCGCAAGCTTTTATCTATACGCTTGAATCAAC	*G .mellonella*
Hemocyanin-C	HaHeCBamHI/F	GGACTGTTCCAAATGTGG	*H. armigera*
Hemocyanin-C	HaHeCHindIII/R	ATGCTTGTGGTAGATCAAGAC	*H. armigear*
Hemocyanin-M	HaHeMBamHI/F	ATTTACATCCCTCGCCTT	*H. armigera*
Hemocyanin-M	HaHeMHindIII/R	TTTCTCCTGCCACATTTG	*H. armigera*

Table 2-12 Primers for PCR screening with pQE vector

Name	primer
pQE-Rev	CATTACTGGATCTATCAACAGG
pQE-For	GAATTCATTAAAGAGGAGAAA

Table 2-13 Predicted parameters for the three domains based on the known DNA sequences

Domain Name	DNA Size	No. of amnio acid residue	Predicted size	pI	Ch
VWD	471bp	157	17.4 kDa	5.97	-2.51
Adhesion	1317bp	439	49.7 kDa	5.03	-14.06
Hemo-C	783 bp	261	29.8 kDa	6.03	-5.43
Hemo-M	828 bp	276	32.6 kDa	6.02	-6.93

2.7.2 Protein induction, extraction, and purification

After determination that the correct sequence was present by PCR, the bacterial colonies containing the insert were expressed and purified according to the manufacturer's protocol (Qiaexpress®). Once the corrected inserts were selected, they were expressed in the vector by induction with 1 mM IPTG for certain time periods. The optimal time for expression was determined by time-course experiments. The colonies were grown in LB with Ampicillin and Kanamycin selection overnight (about 16 h) at 37°C. Bacteria were diluted (50 X) with LB, and grown for an additional 2 h to reach to the exponential growth stage. A negative control was set up using 1 mL of medium without induction by 1 mM IPTG. Samples (1mL) were collected at time points of 1h, 2h, 3h and 4h. The collected samples were centrifuged for 1 min at 13,000 x g. The supernatant was discarded and the pellet was suspended in 100 µl of 1 X SDS-PAGE loading buffer and heated at 95°C for 5 min before separation on 12% SDS-PAGE. Half of the gel was stained with Commassie Blue and the other half was bloted to NC and stained with the poly-histidine antibodies. The sizes of the expressed proteins were larger than those of the predicted proteins (Table 2-13) which is probably due to additional His residues.

The expressed proteins were sonicated in lysis buffer (50 mM Na-phosphate pH 7.8 and 300 mM NaCl), then centrifuged at 13,000 x g for 1 min and the supernatant collected (Extract A). The pellet was dissolved in the same lysis buffer (Extract B). The protein extracts were analysis by SDS-PAGE and Western blots using antibodies (Towbin et al., 1979). Most of the fusion protein will be observed in extract A, if the protein is soluble, and in extract B if it was insoluble. The recombinant protein and was produced by a large-scale expression method and eluted from Ni-beads complex

(Qiaexpress®) using 6 M guanidine hydrochloride. The pQE30 vector was also expressed and purified with the same method and used as a negative control. The elutes were dialysed in 1X TBST at pH 7.5 for two days at 4°C then used for the respective experiments

2.8 Antisera against the recombinant protein fragments

Once the recombinant proteins of interest were purified, they were used for raising antisera in rabbit. Antisera against protein fragments covering the hemocyanin C and hemocyanin M domains from hexamerin of *H. armigera,* and vWD from *G. mellonella* apoliophorin were used as described by (Harlow and Lane, 1998). The elution of specific antibodies binding to the proteins of interest on a Western blot was performed as described by (Smith and Fisher, 1984).

2.9 Functional study of genes of interest

2.9.1 RNAi to test *in vivo* function of hexamerin

To test the *in vivo* function of hexamerin, an RNAi experiment was performed. This experiment included the following parts.

RNA Synthesis

The target RNA was synthesed by using a plasmid, which contained part of the gene in pGEM-T easy vector as template. Before synthesis of the complementary RNA strands with T7 and SP6 polymerase individually, the plasmid was first digested with restriction enzymes to remove the binding sites for SP6 and T7 respectively. The digested plasmids were then extracted from the gel and the RNA polymerase reaction

initiated. The two single RNA strands were mixed and annealed to produce double stranded RNA and stored at –80˚C for later use.

RNA injection

To inactivate the RNA of a gene of interest, the dsRNA is injected into insect larvae at different developmental stages. Preliminary result showed that at early stages the larvae is very fragile and easy to kill by the injection. Therefore, older larvae were chosen to facilitate the injection. Injection with PBS was used as a negative control. The RNAs were extracted and the mortality was recorded 48 hours after injection

Northern Blot

Northern blots were performed based on the methods of (Wu et al., 1997).

RNA preparation

10 µl of formamide was added to cool dried 5-10 µg RNA, then mixed with four µl of formaldehyde, two µl of 10X MOPS buffer, and loading buffer (Appendix 3.4). The mixture was heated at 65˚C for 10 min, spin briefly to collect condensate, and was sit on ice

Preparation of RNase free gel

The tank units were soaked in 1% SDS overnight. A 1.2% agarose gel was poured in a hood to avoid formaldehyde vapours. The agarose was cooled to approximately 60ºC for 3 min in a water bath. Then, 5 ml of 10 X MOPS and 2.9 ml of 37% formaldehyde was quickly added

Electrophoresis

The gel should be loaded and run in the hood. Electrophoresis at 5 V/cm maximum in 1X MOPS buffer until the running dye ¾ of the way down the gel and a photo was took to document.

Blot RNA from gel to nitrocellulose filter

The gel was rinsed in autoclaved water with gentle agitation for 15 min. Then, immersed in autoclaved 20 X SSC for 30-45 min with gentle shaking, use 200ml for every 100 ml of gel. Standard capillary blot was set up to initiate the blotting. (Make a platform and cover it with a wick made from three sheets of Whatman 3MM paper, saturated with blotting buffer (20X SSC)).

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The gel was placed on the wick (upside down). Surrounded with plastic to prevent short circuits of blotting buffer. The gel was overlayed with dry Hybond^{+} membrane. 3 sheets of 3MM paper were covered and soak in the blotting buffer. A stack of paper towels was covered on the top of filter paper. Transfer was proceeded for about 16 hours at RT. RNA blot was fixed with UV-linking.

Probe preparation

Hemocyanin C domain DNA fragment was obtained by PCR. PCR product was labelled with P^{32} according to the method of (Wu et al., 1997).

Prehybridization and hybridization

The membrane was fixed by using the UV chamber (GS GENE LINKER^{TM}), then soaked in blocking buffer (Appendix 3. 4), which was pre-warmed to 65˚C and used to block the membrane for at least 2 h. The probe was added to the block buffer to initiate hybridisation for 16 hours

Development

The blot was washed two times for 15 min with 2xSSC+0.1% SDS at 65 ˚C. Followed by two times of 15 min wash with 0.2xSSC+0.1% SDS at 65˚C. Then the blot was sealed with plastic wrap and put in a cassette. The same size of Kodak film was layered and tagged on the top of the blot for a specific time at –80°C according to

the radiation strength. The film was developed by a Curix 60 (AGFA, CP1000) X-ray film developer.

2.9.2 Enzymatic analysis

2.9.2.1 Alkaline phosphatase activity

Since a difference was noticed between resistant (ISOC4) and susceptible (ANGR) insects in alkaline phosphatase-conjugated control blots in the absence of secondary antibodies, we discovered that alkaline phosphatase from BBMVs retain their enzymatic activity after SDS-PAGE and Western blotting. This protein was therefore visualised directly with phosphatase substrates on the blot.

2.9.2.3 An-enzyme-linked lectin Assay (ELLA) for Gly-T

Gly-T activity was performed according to the method proposed by (Khraltsova et al., 2000). 100 µl of 10µg/ml LacNAc-PAA (20%) was added in Na-carbonate buffer, pH 9.6 in each well. The plate was incubated at 37°C for 16 h to dry the solution. 150 µl of 5% BSA in PBS was added to block the surface. Followed by incubation at 37°C for 1 h, and three washes with PBST. 50 µl of UDP-Gal (at different concentration) in 0.1 M Na-cacodylate buffer, pH 6.8, was added to enable enzymatic glycosylation. Add 50 µl of BBMV preparations from ANGR and ISOC4 in the same buffer at different concentration to initiate the enzymatic reaction. 50 µl of buffer without BBMV preparations as a control. Then incubated at 37°C for 1 h. The solution was remove and washed with PBST. 100 µl of labelled lectin (1µg per ml in 0.3% BSA in PBS) was added and incubated at 37°C for 1 h. The wells were thoroughly washed with PBST to remove unbound lectin. The absorbance at 492 nm measured. Three replicates were performed. Results significance was tested by ANOVA and expressed as mean ± SD.

2.9.3 Bt binding studies

2.9.3.1 Sugar effects on Bt binding

This was performed according to Sarah et al (1999). 5 μg of activated Cry 1Ac was incubating with 50μg of BBMVs from ANGR and $ISOC_4$ at RT for 60 min. Centrifuged at 13,500g for 10 min at RT. The pellet was washed with PBST for 2 times. Both supernatant (S/N) and pellet were subjected to 12% SDS-PAGE and transferred to NC. The NC was stained with anti Cry1Ac antiserum and developed as the method in section 2.5.4.4.

Chapter 3 Results

Bt-resistance was selected in a *H. armigera* population from the field by feeding the toxin in artificial diet in the laboratory. The resulting resistance was based on a single semi-recessive mutation (Akhurst et al., 2003). A resistant iso-genic line ISOC4 was obtained after repeated backcrosses with a susceptible strain (Bird and Akhurst, 2004). Binding assays showed differences in an unknown protein, while none of the known aminopeptidase N genes showed any mutation that could be responsible for resistance (Angelucci et al., 2002). Moreover, exposure to Bt toxin imposed a fitness cost, which is probably derived from pleiotropic effects of the resistance gene rather than effects from other gene loci (Bird and Akhurst, 2004). This suggests that a possible inactivation of receptors other than the known aminopeptidase N proteins was responsible or the existence of resistance mechanisms. Alternatively, the cause of resistance may not be based on receptor inactivation, but on inducible mechanism of pesticide tolerance.

Resistance to high dose cry toxins is usually inherited as a single semi-recessive allele in *H. armigera*. The low dose resistance based on dominant or semi-dominant mode of inheritance is less well understood. To determine the mode of resistance a series of crosses between resistant line (ISOC4) and a susceptible line (ANGR) was undertaken.

3.1 Inheritance of Bt-tolerance in *H. armigera* by a maternal effect

It is possible that an inducible tolerance mechanism is transmitted to subsequent generations by a maternal effect. In principle, reciprocal crosses of resistant and susceptible individuals will produce two different outcomes depending on the mode of transmission of the phenotypic trait. If resistance is caused by a recessive mutation expressed in developing offspring, F1 neonates will be susceptible. In contrast, transmission by a maternal effect is expected to produce resistant offspring from resistant females, while those from resistant males will be susceptible. The two scenarios are depicted below to demonstrate the phenotypic differences of the two possible outcomes. In a putative sex-linked transmission, the incidences of resistance in F1 offspring (in bold) are expected in opposite crosses and restricted to females, since females are hemizygous in lepidopteran species. If resistance is due to a recessive trait, resistance is only detected if the gene is sex-linked (female offspring in the cross shown on the right):

$\mathbf{X^rY}_F \times X^sX^s_M$ $\qquad$ $X^sY_F \times \mathbf{X^rX^r}_M$

F1: $X^rX^r_M$; X^sY_F $\qquad$ F1: $X^sX^r_M$; $\mathbf{X^rY}_F$

If resistance is based on maternal transmission, all offspring of resistant females (cross shown on the left) will be resistant. Similar outcomes are expected whether transmission occurs by maternal inheritance (e.g. mitochondrial gene mutation) or maternal imprinting.

$R(\mathbf{XY})_F \times S(XX)_M$ $\qquad$ $S(XY)_F \times R(\mathbf{XX})_M$

F1: $R(\mathbf{XX})_F$; $R(\mathbf{XY})_M$ $\qquad$ F1: $S(XX)_F$; $S(XY)_M$

3.1.1 Analysis of genetic crosses

One possible mode of transmission could be the incorporation of maternal immune-components into the egg, acting as pro-coagulants in the larval gut or as immune-elicitors during embryogenesis and thus priming the neonates to acquire an elevated immune-status. If this assumption is correct, genetic crosses between resistant and susceptible insects should not be reciprocal; females from resistant populations mated with susceptible males will produce immune-induced offspring, whereas resistant males and susceptible females will produce susceptible offspring. In contrast, if resistance were based on an embryonic phenotype caused exclusively by zygotic gene expression in the embryo, resistance in F_1 offspring would only be expected if the resistance gene locus were linked to a sex chromosome, or was semi-dominant.

Analysis by Cox's proportional hazard model showed that the hazard ratio was significantly lower for the RxS larvae compared to the SxR larvae (Efron, 1977) (Table 3-1A), and correspondingly the contribution of the resistant female was greater than the resistant male (Table 3-1B). These results are consistent with a maternal effect resulting in the transmission of an elevated immune status from the female to her offspring. The fact that the risk ratio for RxR was lower than for RxS and that the risk ratio for SxS was higher than SxR indicates a nuclear gene contribution to the phenotype in addition to the maternal effect.

3.1.2 Sex-linkage study

To test wether the Bt resistance is sex-linked or not, the sex of the surviving larvae was inspected. Results showed that there is no significant difference between female and male survival (Table 3-2). Sex proportion of offspring population from the susceptible female cross is 50 to 50% for both sexes. In the offspring from resistant

female cross, 52% is female whereas 48% is male. This suggested that Bt tolerance in *H. armigera* is not affected by the sex of the larvae.

Table 3-1 Hazard ratios from a 7-day Bt-bioassay, determined by Cox's regression using Efron's partial likelihood method, with the SxS cross treated as the baseline hazard (i.e. hazard ratio of 1). **A)** analysed for the crosses of resistant and susceptible *H. armigera* strains. **B)** analysed for male and female of the resistant *H. armigera* strain (Efron, 1977).

A

Cross (Female x Male)	Hazard Ratio	95% CI	P
RxR	0.041	0.026 - 0.067	<0.0001
RxS	0.183	0.134 - 0.25	<0.0001
SxR	0.554	0.442 - 0.694	<0.0001

B

Sex	Hazard Ratio	95% CI	**P**
Male	0.472	0.368 - 0.577	<0.0001
Female	0.131	0.101 - 0.170	<0.0001

Table 3-2 Surviving pupae from F_1 population exclude the possibility of sex-linkage to Bt tolerance in *H. armigera*.

F_1 Offspring	Female	Male
SXR	50%	50%
RXS	52%	48%

3.2 Hexamerin is induced by a sublethal dose of Bt toxin

Since the genetic transmission of Bt resistance in *H. armigera* is mainly via a maternal effect under low dose toxin exposure, we wanted to know the biochemical basis for Bt resistance in *H. armigera*. Because transmission occurs through a maternal effect, the biochemistry for Bt resistance in this strain must be distinct from the well-known "Mode I" mechanisms. In gut preparations from resistant strains, the peritrophic membrane and the gut content were frequently darker and in some extreme cases showed large black patches of melanisation products, whereas the susceptible gut was always transparent and white (Fig. 1-4). The blackening gut implied that Bt resistance might be associated with immune induction. Therefore the potential for an elevated immune response to contribute to Bt resistance was tested.

3.2.1 Melanisation in the hemolymph and gut lumen

To examine a possible association between Bt-resistance and an elevated immune status in the resistant strain, we measured the melanisation reaction in cell-free hemolymph (plasma) from the resistant strain as a first approximation of an induction of the humoral immune system (Shelby et al., 2000; Soderhall and Cerenius, 1998).

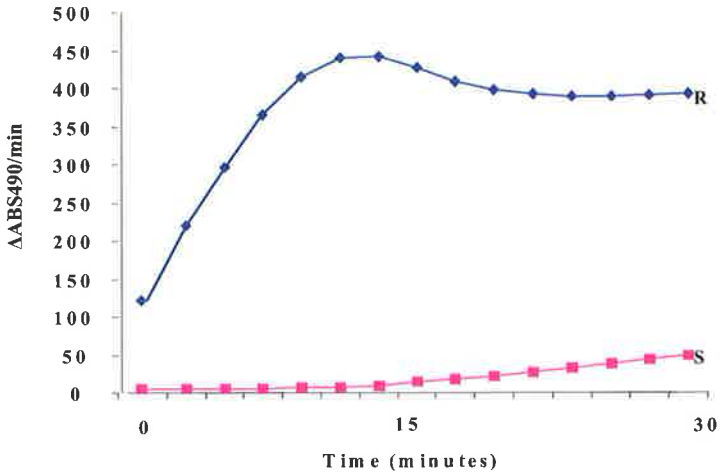
When plasma of larvae from the resistant strain was analysed, a strong melanisation reaction was observed within one hour, whereas no significant melanisation was observed in plasma from susceptible larvae (Fig. 3-1A). When gut extracts from resistant and susceptible larvae where examined in the presence of DOPA, higher melanisation rates were observed in the gut extracts from resistant compared to susceptible larvae (Fig. 3-1B).

3.2.2 Hemolymph proteins are induced

To examine whether immune induction causes changes in the protein abundance in the hemolymph, the protein patterns of plasma from resistant and susceptible larvae was compared. For resistant larvae most of the major plasma proteins were significantly reduced in amounts relative to the susceptible larvae, even when plasma was collected on ice and dissolved in anti-coagulant buffer (not shown). The reasons for this reduced recovery of hemolymph proteins is not clear, but may be due to an increased reactivity of pro-coagulants.

In plasma from non-induced susceptible larvae, several minor proteins in the range of 85-90 kDa that bind to Bt-toxin (not shown) and GalNAc-binding lectins were observed (Fig. 3-2). One of these has antigenic similarity to apolipophorin I (not shown), and could be a proteolytic digestion product of intact apolipophorin I. Following induction by various elicitors, including sub-lethal doses of Bt formulation, plasma from both strains displayed a dramatic increase in the level of an 85 kDa protein (Fig. 3-2). Together with the observed differences in melanization reactions (Fig. 1-4 and 3-1A), this suggested an increased coagulation reaction in the plasma of resistant larvae.

A



B

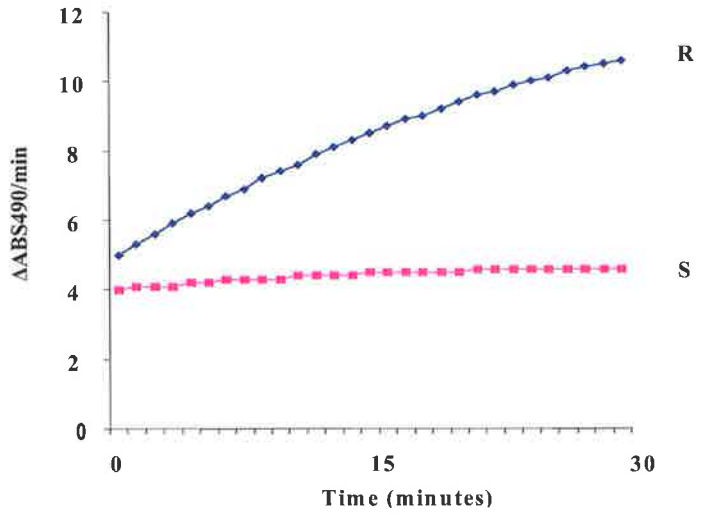


Fig. 3-1 Typical melanization reactions measured as the relative absorbance at 490 nm over 30 min. A) Cell-free hemolymph (plasma) from groups of five 3rd instar resistant and susceptible caterpillars diluted in PBS-solution. Note the slight reduction in relative absorbance observed in plasma from the resistant strain after 10 mins due to partial coagulation reactions. B) Gut protein extracts from resistant and susceptible caterpillars diluted in PBS-solution containing 100mM DOPA.

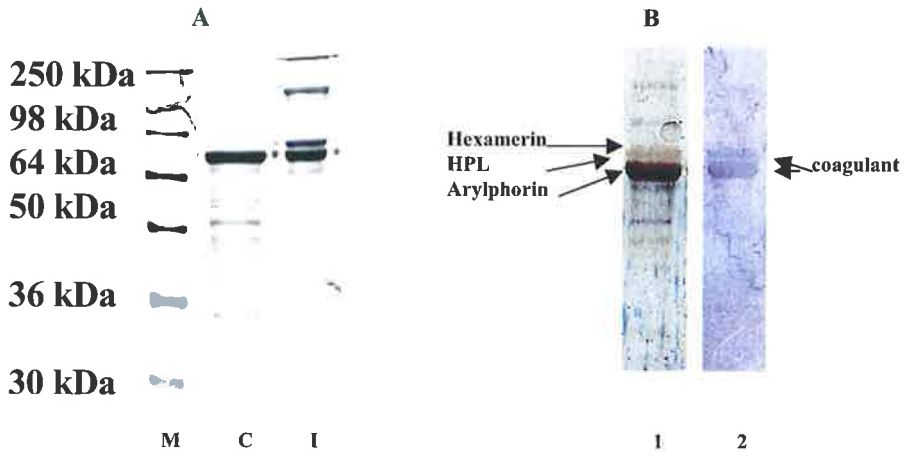


Fig. 3-2 SDS polyacrylamide gels of cell-free hemolymph (plasma) from induced (I) and non-induced (C) larvae. **A**) Gel stained with Coomassie blue. A protein band at 85 kDa is visible in plasma from induced larvae, in addition to a band at around 160-180 kDa that is not always visible and may constitute a dimer. **B**) In **lane 1**, Western blot incubated with peroxidase or phosphat-conjugated GalNAc-specific HPL (HPL definition is in Table 2-3, p29) and anti-arylphorin antibodies visualised with peroxidase-conjugated secondary antibodies. The protein band labelled by anti-arylphorin antibodies is comprised of three proteins with similar sized apolipophorin II and prophenoloxidase proteins in addition to arylphorin. In **lane 2**, the blot was stained with serum against coagulant from *G. mellonella* (Li et al., 2002) indicated that the induced protein is a possible pro-coagulant.

3.2.3 p85 and LPS-mediated aggregation *in vivo*

In plasma from non-induced susceptible larvae, several minor proteins in the range of 85-90 kDa are observed with Bt-binding activity. Since one of these may be an immune protein, immune-induction was studied by using sublethal dose of Bt toxin in the food.

Following induction by various elicitors, including sub-lethal doses of Bt-formulation, plasma from both strains displayed a dramatic increase in the level of an 85 kDa protein (Fig. 3-2A). Since the p85 binds to GalNAc-specific lectins (Fig. 3-2B) and Cry1Ac on Western blots (not shown), whether this binding is also observed with native proteins was examined. Plasma from induced larvae was mixed with activated Cry1Ac and separated into precipitating aggregates and soluble supernatant. When pellets were re-suspended and treated in various loading buffers at different temperatures, no soluble protein was recovered (not shown). Therefore, the protein patterns in the supernatant for the absence of proteins that may have interacted with Cry1Ac to form insoluble aggregates was analysed. When protein extracts of plasma supernatants were analysed on SDS-PAGE, a concentration and time-specific reduction of p85 was observed in Cry1Ac-treated plasma, while the other major proteins, including prophenoloxidase, apolipophorin II and arylphorin were unaffected (Fig. 3-3).

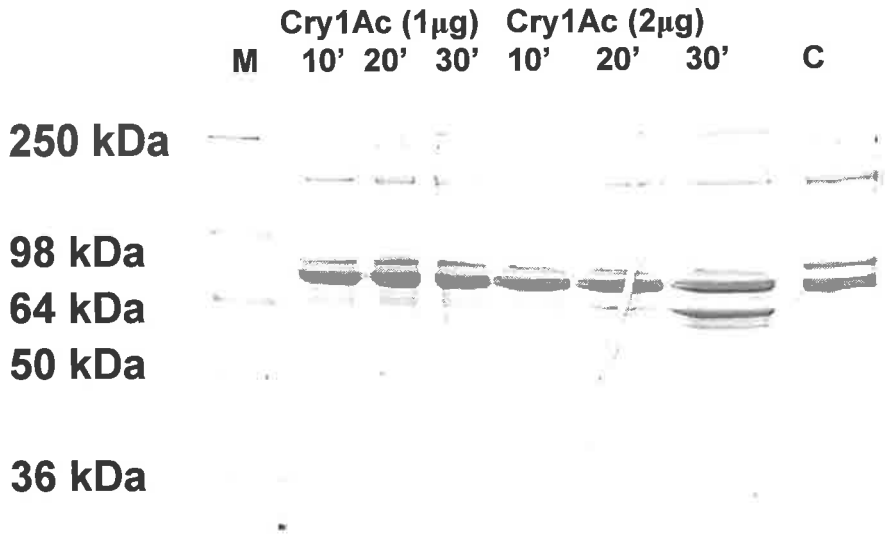


Fig. 3-3 Toxin forms insoluble aggregates with hemolymph protein. SDS polyacrylamide gel, stained with Coomassie blue, of cell-free hemolymph (plasma) from induced larvae incubated with proteolytically activated Cry 1Ac for various time periods. Since the toxin formed insoluble aggregates, the plasma supernatant was analysed for the absence of proteins. In this experiment 1 µg and 2µg of activated Cry1Ac was incubated with plasma for 10, 20 and 30 min at RT, then centrifuged for 1min at 12000rpm. PBS treated plasma is shown as a negative control. Note that the triple protein band at 75 kDa is unchanged, whereas the amount of p85 is reduced. There is also a slight reduction in the band at around 160-180 kDa, which could be a dimer of p85. The newly emerging band(s) around 64 kDa is proteolytically cleaved PO.

Since p85 stained with anticoagulant serum (Fig. 3-2B), it is possible that p85 is a procoagulant and has a potential function in the coagulation process (Scherfer et al., 2004) Since p85 binds to the hexameric GalNAc-specific lectin from *Helix pomatia* (HPL) (Fig. 3-2B), it is possible that HPL and other lectins also induced aggregate formation. When plasma from induced larvae was mixed with Cry1Ac and lectins, only GalNAc-specific lectins (tetrameric *Vicia vilosa* (VVL) and hexameric HPL) caused aggregate formation, whereas the Gal-specific peanut agglutinin (PNA) had no effect (Fig. 3-4). Moreover, the Gal/GalNAc-specific winged bean lectin (WBL), which is probably monomeric under the pH-conditions used, also showed no aggregation (Fig. 3-4).

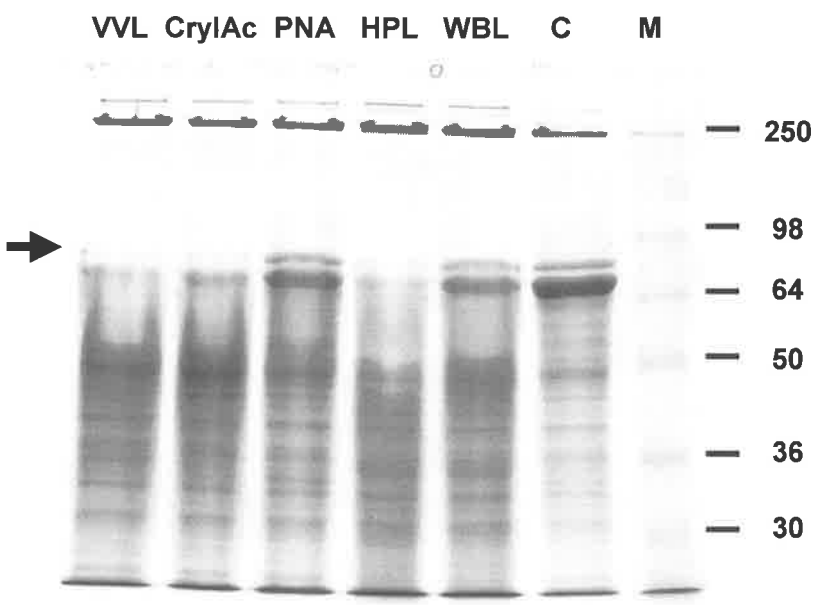


Fig. 3-4 Toxin and lectins interact with hemolymph p85 SDS-PAGE (12% gel) stained with Coomassie blue of ca. 5µg aliquots of cell-free hemolymph (plasma) from induced larvae incubated with various lectins and with Cry1Ac, showing enhanced aggregation and precipitation of hemolymph components, including p85, after incubation of plasma with oligomeric GalNAc-specific lectins and Cry1Ac. VVL=*Vicia vilosa*, a tetrameric GalNAc-specific lectin; Cry1Ac (gut juice-activated Bt-toxin); PNA=peanut agglutinin, a tetrameric Gal/GalNAc-specific lectin; HPL=*H. pomatia*, a hexameric GalNAc-specific lectin; WBL=winged bean lectin *Psophocarpus tetragonolobus,* a monomeric GalNAc-specific lectin; C=PBS control. Addition of gut-juice extracts at a concentration equivalent to those used in toxin activation did not have an effect and proteins were similar to the PBS control (not shown). In this preparation little or no 160-180 kDa protein was detected. (This experiment was performed with the help of M. Sarjan)

3.2.3 Mature Bt-toxin forms tetrameric complexes *in vitro*

Since these experiments indicated that oligomeric but not monomeric lectins cause aggregation of plasma proteins, it was important to determine whether Bt-toxin exists in an oligomeric form outside lipid membrane-bilayers. When Cry1Ac crystal protein was processed for different time periods in the presence of gut juice extracts, high molecular weight complexes were observed at intermediate time periods. When digestion continued for longer time periods, a 60 kDa protein appeared in addition to the putative mature 69 kDa protein (Fig. 3-5A). Under conditions where mixtures of the two proteins co-existed, the high molecular weight complex was separated into several narrow bands above 250 kDa (Fig. 3-5A, arrows). The relative distribution of these high molecular weight complexes correlated with the relative amounts of the 60 and 69 kDa proteins, which is consistent with the formation of hetero-tetramers of 60 and 69 kDa proteins. Both the complexes and the 60 and 69 kDa proteins were identified by anti-toxin antibodies in Western blots (Fig. 3-5B). The tetrameric complexes were stable in SDS at 65°C but reverted to low molecular weight proteins at 100°C (Fig. 3-5B). A similar complex was observed when the 130 kDa toxin precursor was incubated with trypsin in a lipid-free buffer (not shown). This suggests that Cry1Ac-toxin exists as a tetrameric complex with GalNAc-specific lectin properties, which can interact with soluble glycoproteins to form detergent-insoluble aggregates.

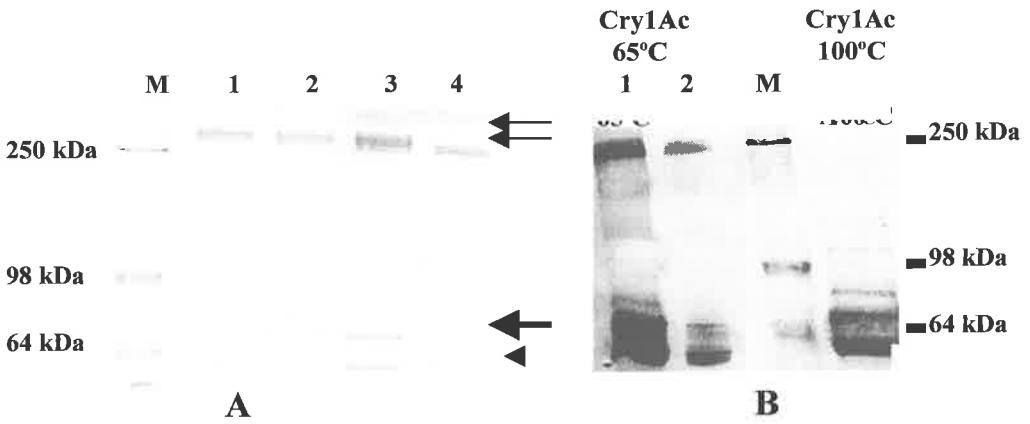


Fig. 3-5 Mature toxin forms a stable tetramer *in vitro*. SDS-PAGE (7.5% gels) of activated Cry1Ac toxin following incubation of protoxin with gut juice extracts for different time periods and extraction at either 65 or 100°C. Protoxin was purified from *B. thuringiensis* subsp. *kurstaki* HD-73 and solubilized in a solution containing 30 mM Na_2CO_3 and 1% mercaptoethanol at pH 9.6. Western blots were incubated with Cry1Ac-specific antibodies and then visualised with peroxidase-conjugated secondary antibodies. **A**) Protoxin and gut juice-extract from the lepidopteran species *Pieris rapae* were incubated for 30 min (1), 1h (2), 2h (3) and five hours (4) and extracted at 65°C in SDS-containing buffer. The mature toxin (69 kDa) is the predominant band initially (large arrow), but is replaced by a 60 kDa over digested protein (large arrowhead). Both proteins appear to form hetero-oligomeric complexes, which form a cluster of narrow bands above the 250 kDa marker band (small arrows). The relative amounts and distribution of these narrow bands are correlated with the relative composition of the 60 and 69 kDa bands. **B**) Gut juice-activated toxin after incubation of one hour (1) and five hours (2), marker (M), gut juice activated toxin (as in 1) extracted at 100°C. Note extraction at 100°C in SDS-containing buffers eliminated the bands above 250 kDa. Cry1Ac-specific antibodies were visualised with peroxidase-conjugated secondary antibodies. (This experiment was performed with the help of N. Feartherstone and M. Sarjan).

3.2.4 Identification of the Cry1Ac-induced protein

Identification of the induced 85 kDa hemolymph protein (Fig. 3-2A) from induced larva of *H. armigera* was attempted by micro sequencing. In-gel protein digestion was followed by separation of extracted peptides on an HPLC column (Fig. 3-6). This produced several peptide peaks and sequences were determined from peaks 14 (elution time 34.2 min), 26 (elution time 44.0 min) and 37 (elution time 53.5 min). Peak 14 was L/H A/T E/P E/H IEVPH, peak 26 was LTDEPEGFPVNRPL, and peak 37 was NIEHYXXVVAVTY.

Analysis of the MALDI-TOF data showed that the protein showed highly significant similarities to a 41-kDa fragment of p82 riboflavin-binding hexamer monomer of *H. virescence* (Noctuid moth). The following sequence tags were determined and the chromatographs are shown from Fig. 3-7 to Fig. 3-10.

DPAFYMIWK VLGLFQMWQEK KEELA**L**PQVAIQK

DPAFYMIWK VLGLFQMWQEK KEELA**M**PQVAIQK

The protein sequence of the p82 riboflavin binding hexamer monomer of *H. virescensis* is

IWRDCLMALV KSPELGHEIV KQGYSSGLLY HNGVPFPVRP IYFNLDQPQF
VNEIQEILDY ERRIRDAIDQ GYVVNHLGEH IDICAPEAIE ILGNLIEANV
DSPNGKYYKD FISIWKKLLG NSIVQEQQYH NNYVPLVVPS VLEHYQTALR
DPAFYMIWKR VLGLFQMWQE KLLLYKKEEL AMPQVAIQKV DVDKLMTYFE
HTYLNVSSHL HMNEDEVKEV HDQVRVLVQH PVLNHKKYQV RVHVKSEVAK
TVRVKFFLAP KYDTQGQEIP LHLNTQNFMQ LDEFLYDLPS GECVISRDSV
DTSGKKLMSG NEVYEAVVKA VQGKGHYTIN ETPEKLADHL LLXKGRVGAC
LSS

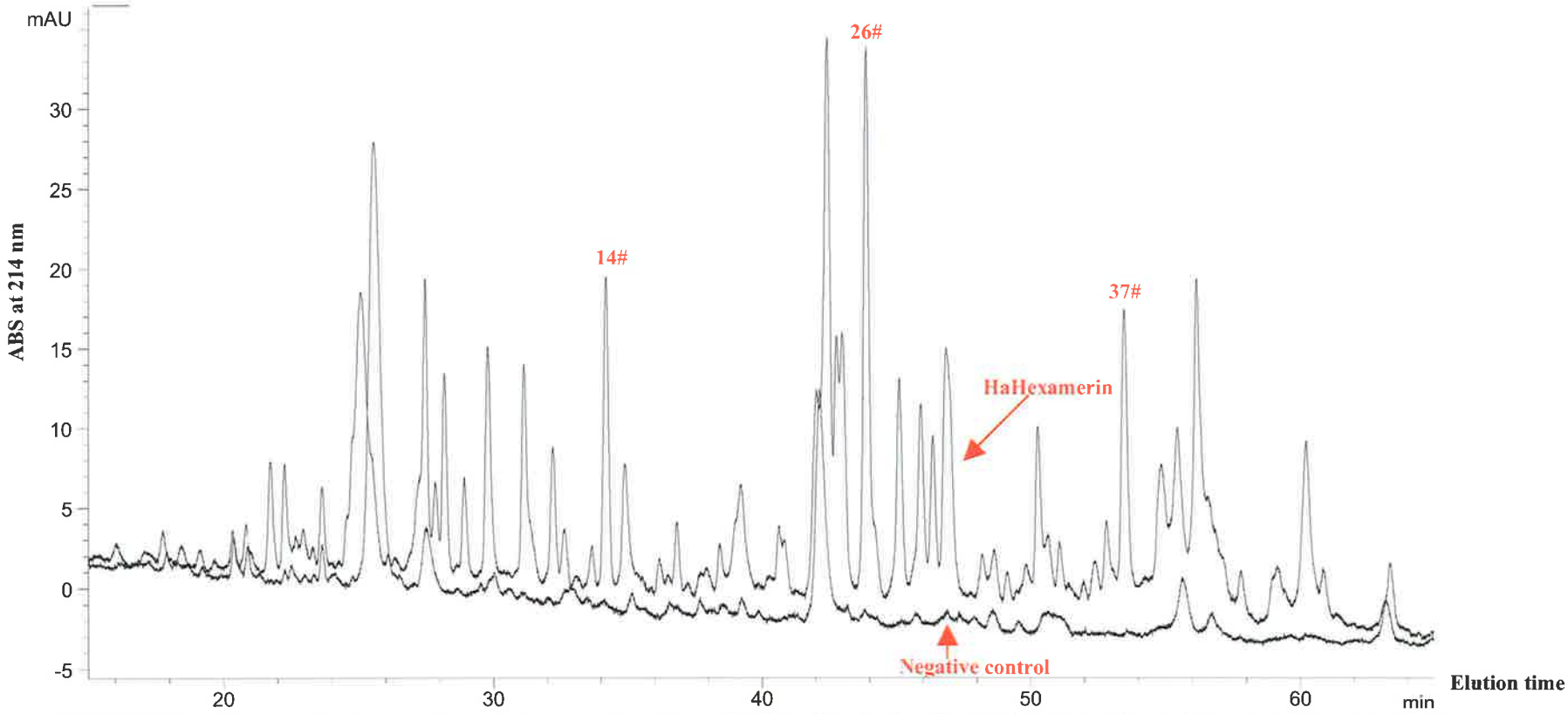


Fig. 3-6 RP-HPLC of the trypsin in-gel digested hexamerin. The unbroken line is the digested hexamerin and the negative control (trypsin-treated protein-free gel piece) is in broken line. Fraction 14, 26 and 37 were selected for subsequent sequencing.

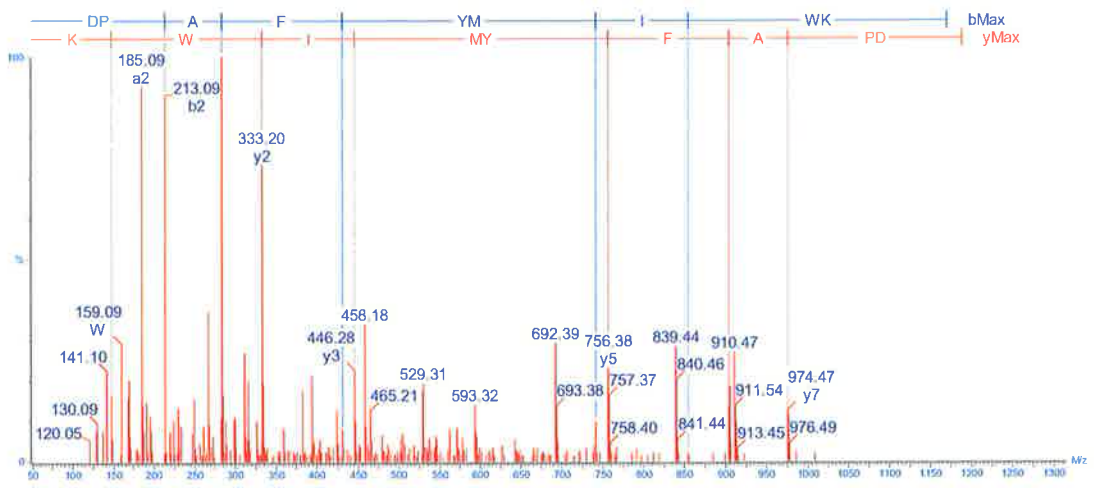


Fig. 3-7 The 1st peptide tag of DPAFYMIWK showed by MALDI-TOF data

(Oxidation of M)

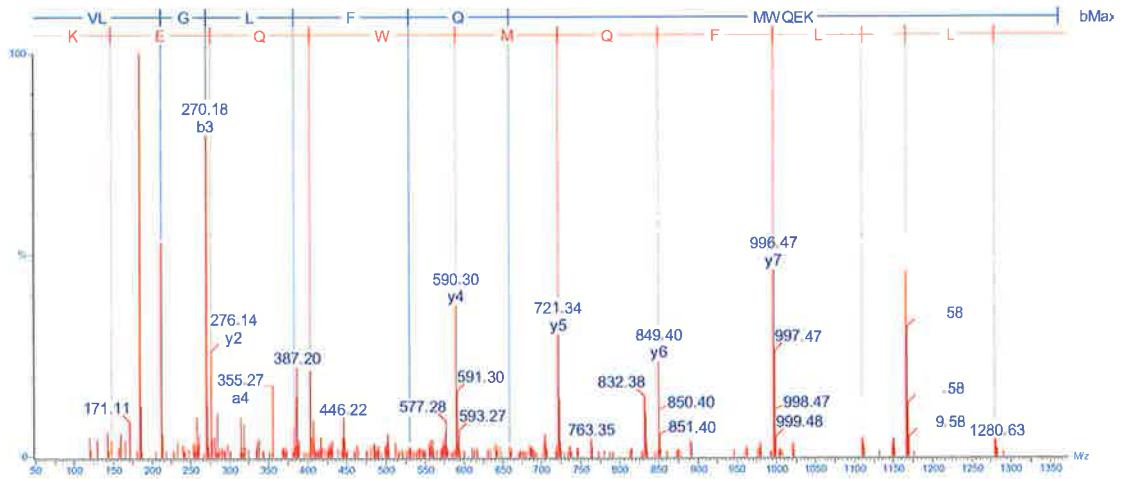


Fig. 3-8 The 2nd peptide tag of VLGLFQMWQEK showed by MALDI-TOF data

(also present as Oxidation of M).

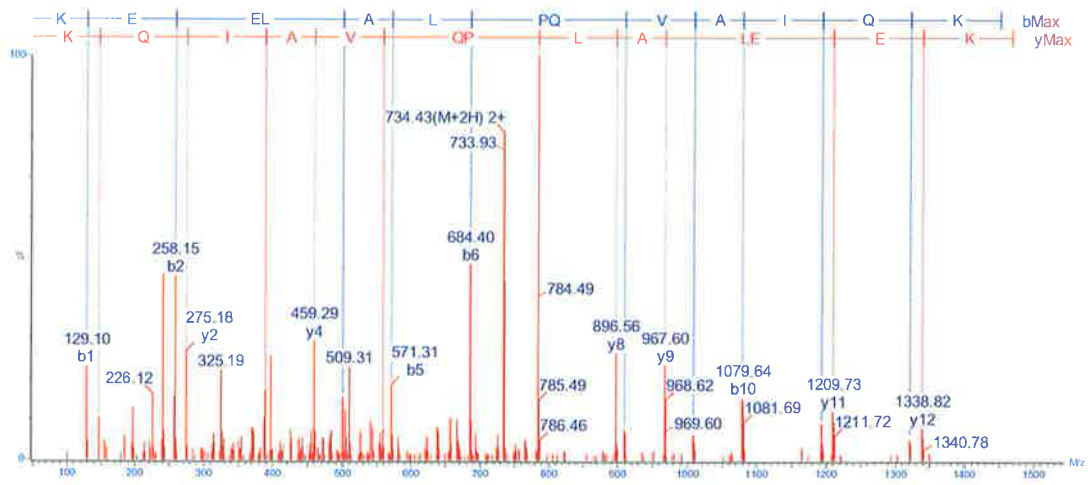


Fig. 3-9 The 3rd peptide tag of KEELALPQVAIQK showed by MALDI-TOF data (Oxidation of M).

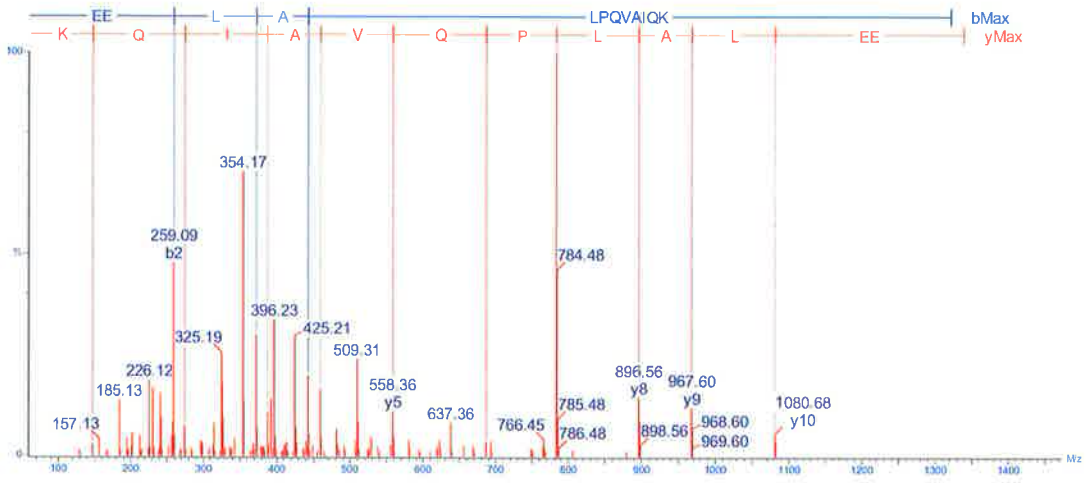
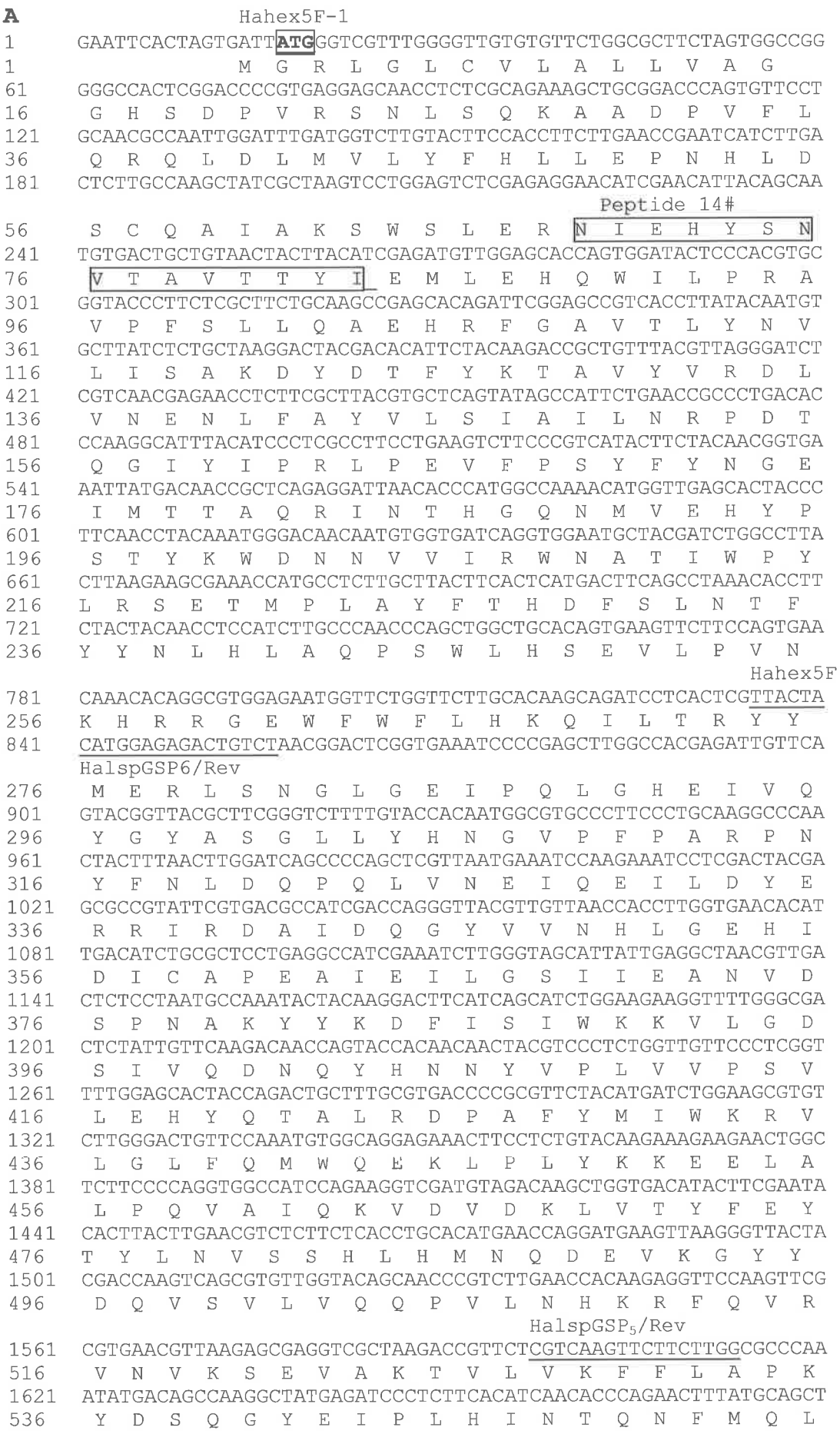


Fig. 3-10 The 4th peptide tag of EELALPQVAIQK showed by MALDI-TOF data (Oxidation of M). The accuracy of the instrument was within 15ppm at the time of the experiment.


A
Hahex5F-1
Peptide 14#
Hahex5F
HalspGSP6/Rev
HalspGSP5/Rev

```
                                                        HalspGSP4/Rev
1681  GGATGAGTTCACGTATGACCTTCCTGCGGGCGAATGCACAATTACTCGTGATTCCATTGA
556    D  E  F  T  Y  D  L  P  A  G  E  C  T  I  T  R  D  S  I  D
1741  CACCTCTGGCAAGAAGTGGGTGTCCGGTATCGAGATATATGAGGCGGTTGAGAAGGCTGT
576    T  S  G  K  K  W  V  S  G  I  E  I  Y  E  A  V  E  K  A  V
1801  GCAAGGCAAGGGACAGTACACCATCGACCCGAACATGGAGAAACTCGCCGAACATCTTAT
596    Q  G  K  G  Q  Y  T  I  D  P  N  M  E  K  L  A  E  H  L  M
1861  GCTGCCTAAGGGTCGCGTCGGAGGCATGCCGTTCGTCCTGATGGTCTACATCTCAGAGTA
616    L  P  K  G  R  V  G  G  M  P  F  V  L  M  V  Y  I  S  E  Y
1921  CCACGCCCCGAAGGTTGCTCCCGAACAAGTCTCGTACCCAGCTTTGTCTCTTGGCCTGTC
636    H  A  P  K  V  A  P  E  Q  V  S  Y  P  A  L  S  L  G  L  S
                                        HalspGSP3/Rev
1981  TCCCGTTATTCGCCAACTGACCGACGAGCCATTCGGTTTCCCAGTCAACAGGCCTCTTCA
                          Peptide 26#
656    P  V  I  R  Q  L  T  D  E  P  F  G  F  P  V  N  R  P  L  H
2041  CCCATGGCAGGTGGAGGGAGTCAAGAACTTGTACCTCCAAGATGTCTTGATCTACCACAA
676    P  W  Q  V  E  G  V  K  N  L  Y  L  Q  D  V  L  I  Y  H  K
                          HalspGSP2/Rev
2101  GCATACCCCCGAAATCGAGGTTCCCCACATGGAATAAGCGATTCTCGGGATGTTATTGAC
      Peptide 37#
696    H  T  P  E  I  E  V  P  H  M  E  O
2161  GCCAGATTTAGAACAATGATACTTCTCCGTGAATAAACTACTGAGCTTCTGGAATGAAGA
2221  TAAGATCCATATGTCCTTTATGAAGCAAGGGCTGATGAACTAGCTCAAATTACTTCAAGC
2281  TCTTCAATTTGGCGTCGACAAACAATTAATGTGCATTTATGTTATGTTCATGTATTATGT
2341  ATTTATTAGTATATGTATTTATTGTTGATGGATATCTTCTTATATGTGTGTATTGCATTA
2401  TGTAGTGAAATATACTATGATGATCAAAAAAAAAAAAAAAAA 2442
```

B

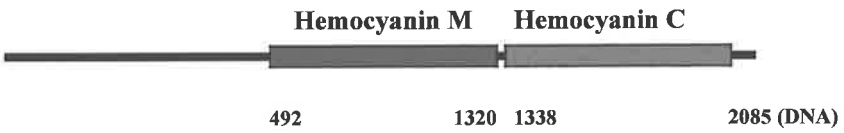
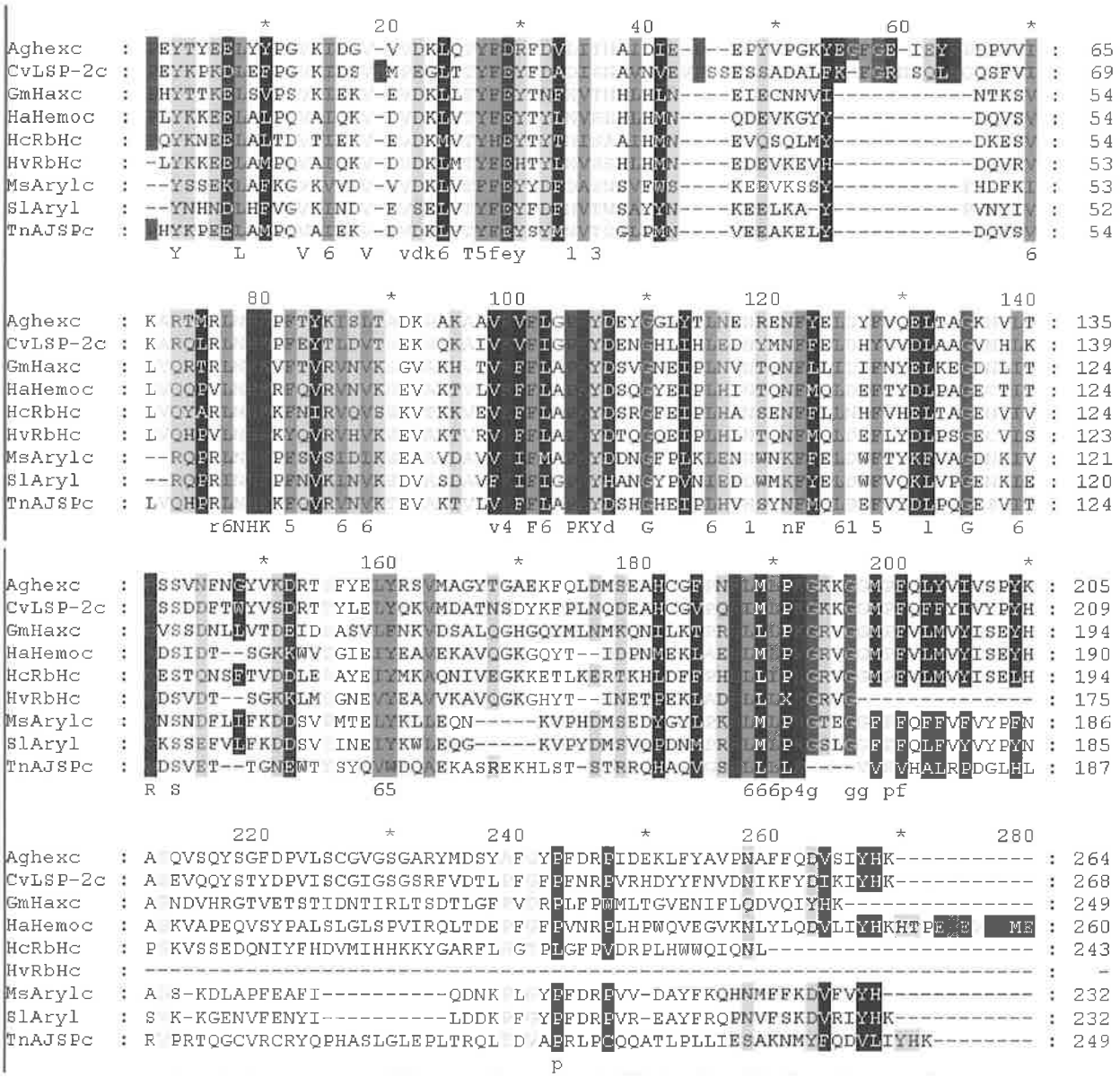


Fig. 3-11 Full-length and domain structure of the hexamerin cDNA sequence from *H. armigera* **A):** The initiation codon **ATG**, stop codon **TAA**, peptide number, primer locations, and poly-A tail are all indicated in the sequence. The nucleotide sequence reported in this paper has been submitted to the GenBank™/EBI Data Bank: The *H. armigera* hexamerin sequence accession number is AY661710. **B):** Domain structure of hexamerin from *H. armigera*. There are two domains, hemocyanin C (red colour) and hemocyanin M (blue colour). The positions of the two domain was expressed by the numbers

3-12A



3-12B

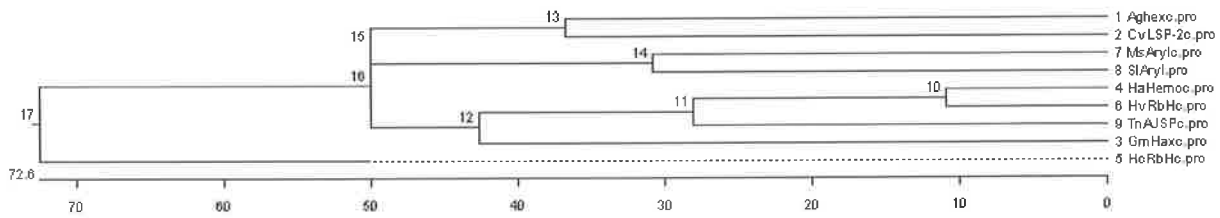
Percent Similarity

	1	2	3	4	5	6	7	8	9	
1		49.2	33.3	31.5	31.7	27.4	34.5	37.5	27.3	1
2	73.5		28.1	28.8	28.0	22.9	35.8	29.7	22.9	2
3	100.0	100.0		50.6	43.2	48.0	27.6	30.2	37.3	3
4	100.0	100.0	65.7		45.7	80.6	30.6	32.8	54.2	4
5	100.0	100.0	90.6	78.7		45.7	23.3	25.0	35.4	5
6	100.0	100.0	75.4	21.9	83.0		30.9	28.6	60.6	6
7	100.0	100.0	100.0	100.0	100.0	100.0		56.0	26.7	7
8	100.0	100.0	100.0	100.0	100.0	100.0	61.7		26.7	8
9	100.0	100.0	100.0	62.2	100.0	49.9	100.0	100.0		9
	1	2	3	4	5	6	7	8	9	

Percent Divergence

1 Aghexc.pro
2 CvLSP-2c.pro
3 GmHaxc.pro
4 HaHemoc.pro
5 HcRbHc.pro
6 HvRbHc.pro
7 MsArylc.pro
8 SlAryl.pro
9 TnAJSPc.pro

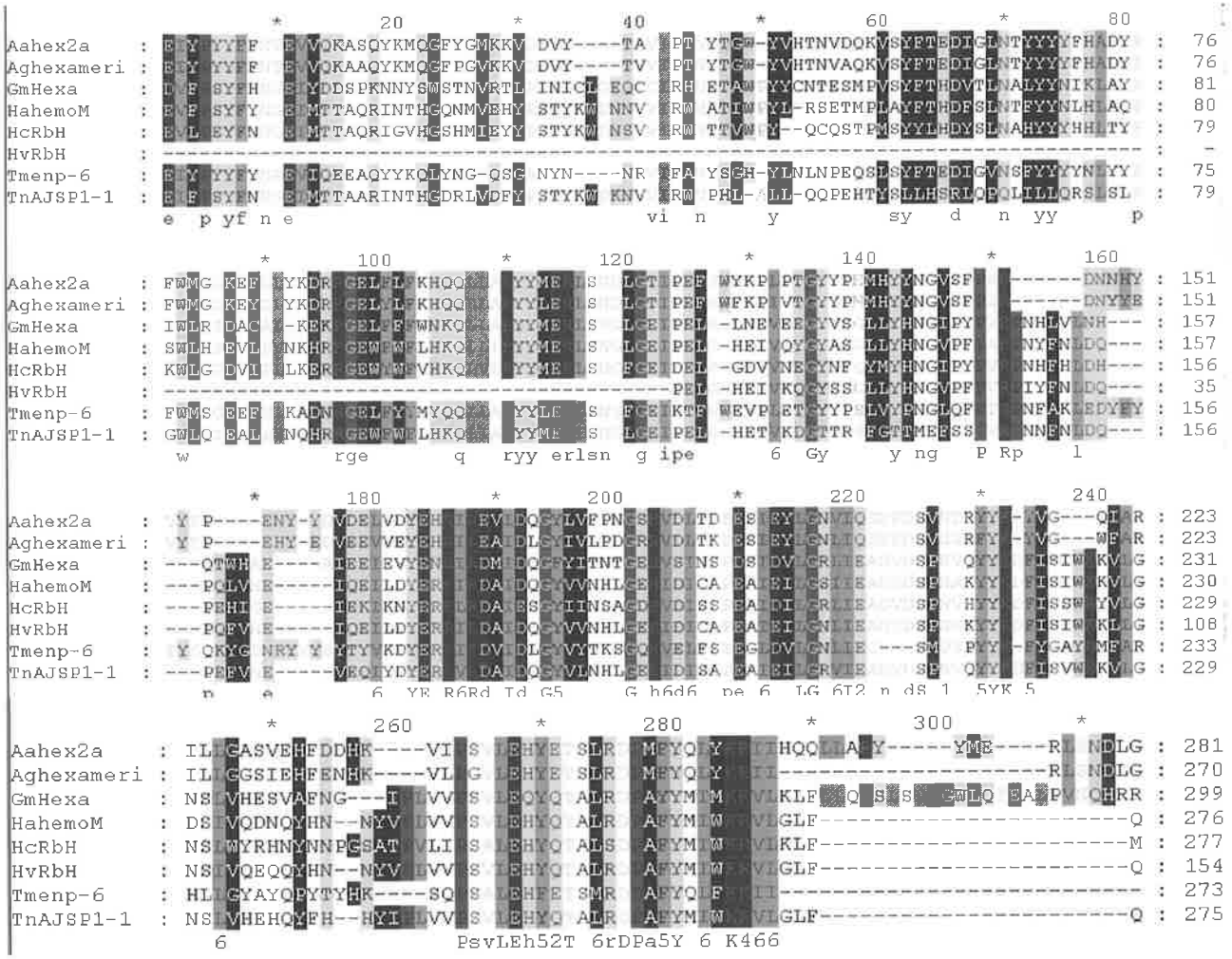
Pair distance of HemocyaninC domain



Phylogenetic tree of hemocyanin C domain

Fig. 3-12 Sequence comparison of hemocyanin C domain of hexamerin from *H. armigera* with others. A) Sequence alignment of hemocyanin C domain from *H. armigera*. The alignment was performed by using "GeneDoc" program (www.psc.edu/biomed/genedoc). The score program is Blosum 62. The scoring matrix is 20, the open gap penalty is 8, and the extend gap penalty is 4. B) Pair distance and phylogenetic tree of hemocyanin C domain (Hahemoc) from *H. armgiera*. Hahemoc has a high similarity to hemocyanin C domain from *H. virences* (80.6%).

3-13A


Aahex2a
Aghexameri
GmHexa
HahemoM
HcRbH
HvRbH
Tmenp-6
TnAJSP1-1

3-13B

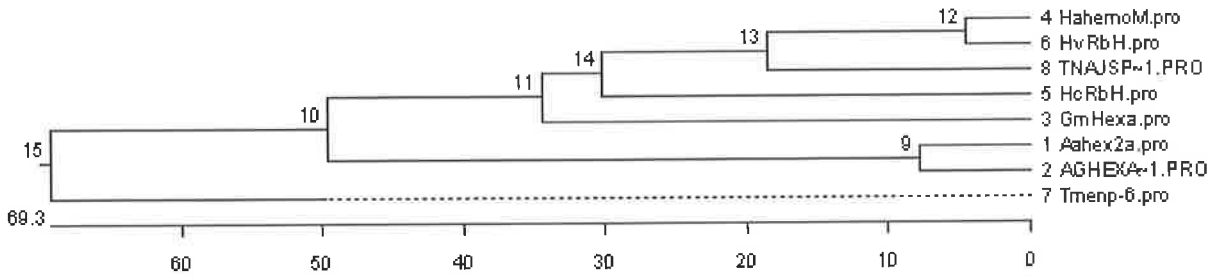
Percent Similarity

	1	2	3	4	5	6	7	8	
1		83.3	30.6	37.3	32.1	39.0	45.1	31.3	1
2	15.6		30.7	37.8	34.8	39.0	45.2	30.7	2
3	100.0	100.0		52.5	49.5	59.7	27.1	44.7	3
4	99.2	100.0	61.8		60.1	91.6	31.9	66.5	4
5	100.0	100.0	69.3	52.2		58.4	28.6	54.2	5
6	92.5	93.9	47.9	9.0	55.8		31.8	74.7	6
7	77.3	78.3	100.0	100.0	100.0	100.0		22.7	7
8	100.0	100.0	81.8	42.9	66.3	30.9	100.0		8
	1	2	3	4	5	6	7	8	

Percent Divergence

1 Aahex2a.pro
2 AGHEXA~1.PRO
3 GmHexa.pro
4 HahemoM.pro
5 HcRbH.pro
6 HvRbH.pro
7 Tmenp-6.pro
8 TNAJSP~1.PRO

Pair distance of HemocyaninM domain



phylogenetic tree of HemoM

Fig. 3-13 Sequence comparison of hemocyanin M domain of hexamerin. A) Sequence alignment of hemocyanin M domain from *H. armigera*. The alignment was performed by using "GeneDoc" program (www.psc.edu/biomed/genedoc). The score program is Blosum 62. The scoring matrix is 20, the open gap penalty is 8, and the extend gap penalty is 4. B) Pair distance and phylogenetic tree of hemocyanin M domain (HahemoM) from *H. armgiera*. HahemoM has a high similarity to hemocyanin C domain from *H. virences* (91.6%).

3.2.5 Cloning of p85 coding DNA

To get the full-length sequence of the hexamerin cDNA from *H. armigera*, a five-step-strategy was employed including 3'RACE, 5'RACE and degenerate RT-PCR (Fig. 2-4). Cloning of the corresponding coding DNA (see Material and Methods) revealed a deduced protein of 706 amino acids with conserved hemocyanin M and C domains (Fig. 3-11).

3.2.6 Alignment of the sequence

The sequences of hemocyanin C and hemocyanin M from several insects were aligned using the "GeneDoc" program (www.psc.edu/biomed/genedoc). The alignment results including paired distance, and phylogenetic trees for both domains are shown in Fig. 3-12 and Fig. 3-13.

3.2.7 Structure prediction based on sequence information

To obtain the protein features of hexamerin from *H. armigera*, the deduced structural characteristics were predicted by using computer programs as described in 2.6.5.3. The first 19 amino acids were identified as a signal peptide with a signal peptide cleavage site at position 18(S) and 19(D) (Fig. 3-14), which predicted an extracellular location with several N-glycosylation sites (Fig. 3-15) and one possible O-glycosylation site (Fig. 3-16). 10 potential serine and 12 tyrosine phosphorylation sites from hexamerin were predicted (Fig. 3-17). A potential coiled-coil between 300 to 400 residues was found in hexamerin (Fig. 3-18).

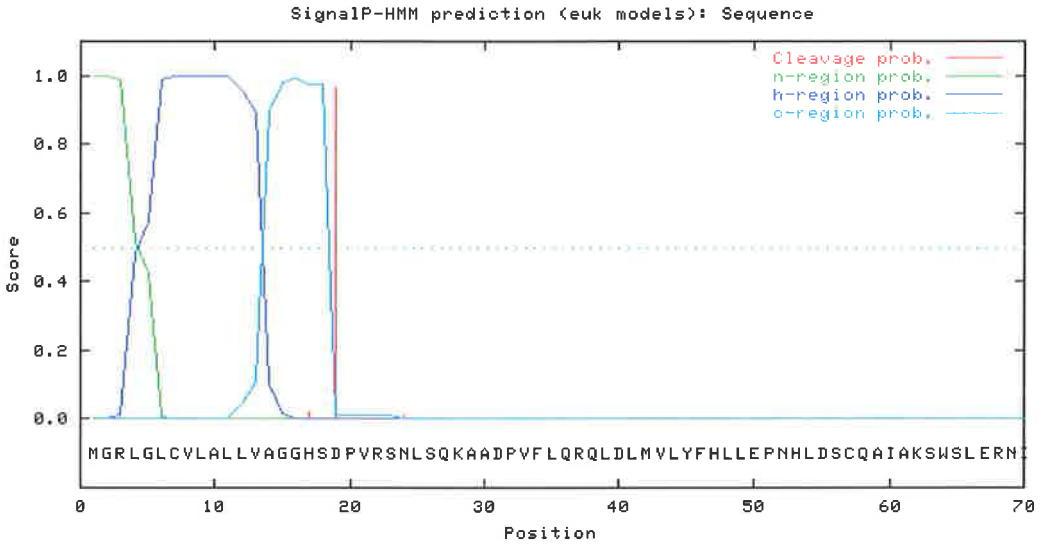


Fig. 3-14 Signal P-HMM result of hexamerin from *H. armigera.* Signal peptide probability is 1.000; signal anchor probability is 0.000; max cleavage site probability is 0.964 . The cleavage site position is between pos. 18 and 19.

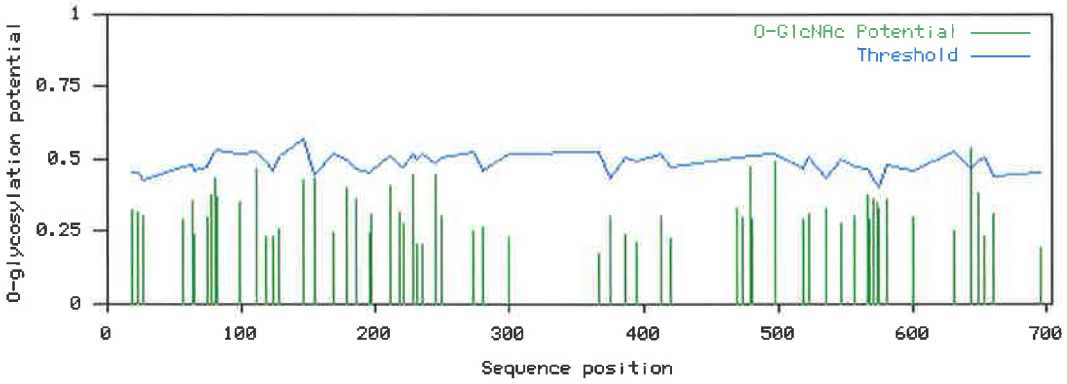


Fig. 3-15 Predicted O-(beta)-GlcNAc sites in hexamerin from *H. armigera* by YinYang 1.2. There is one potential O-glycosylation site in hexamerin.

Residue	O-Glycosylation	Potential	Thresh(1)	Thresh (2)
644 S	+	0.5414	0.4643	0.5762

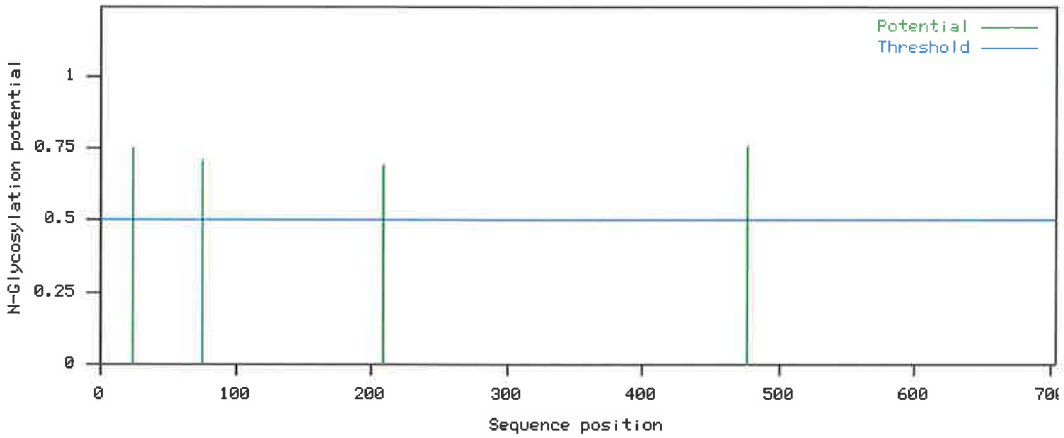


Fig. 3-16 Predicted N-glycosylation sites in sequence by NetGlyc 1.0.There were four potential N-glycosylation sites in the hexamerin from *H. armigera.*

(Threshold= 0.5)

Position	Potential	Jury agreement	N Glycosyl result
24 NLSQ	0.7520	9/9	+++
75 NVIA	0.7087	8/9	+
209 NAII	0.6912	9/9	++
477 NVSS	0.7553	9/9	+++

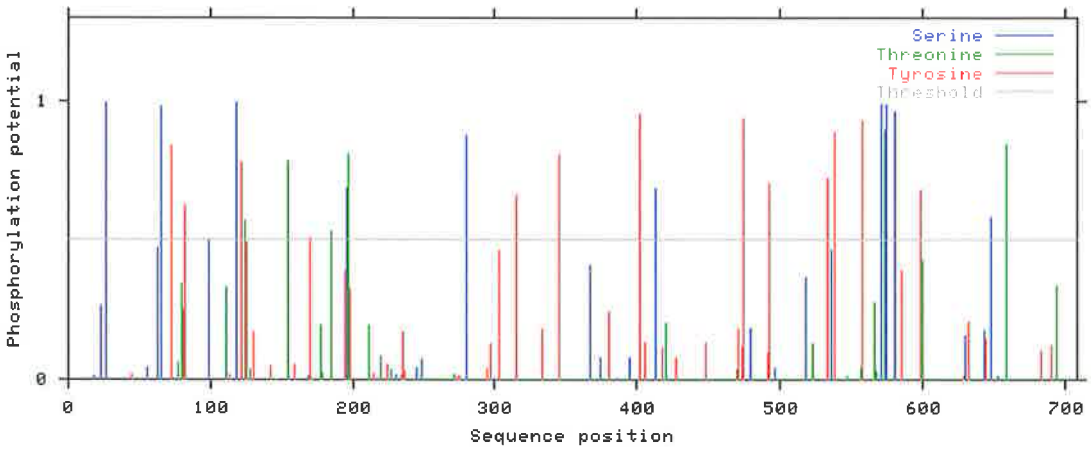


Fig. 3-17 Prediction of phosphorylation sites in hexamerin sequence from *H. armigera* by NetPhos 2.0. There are many potential phosphorylation sites in hexamerin sequence.

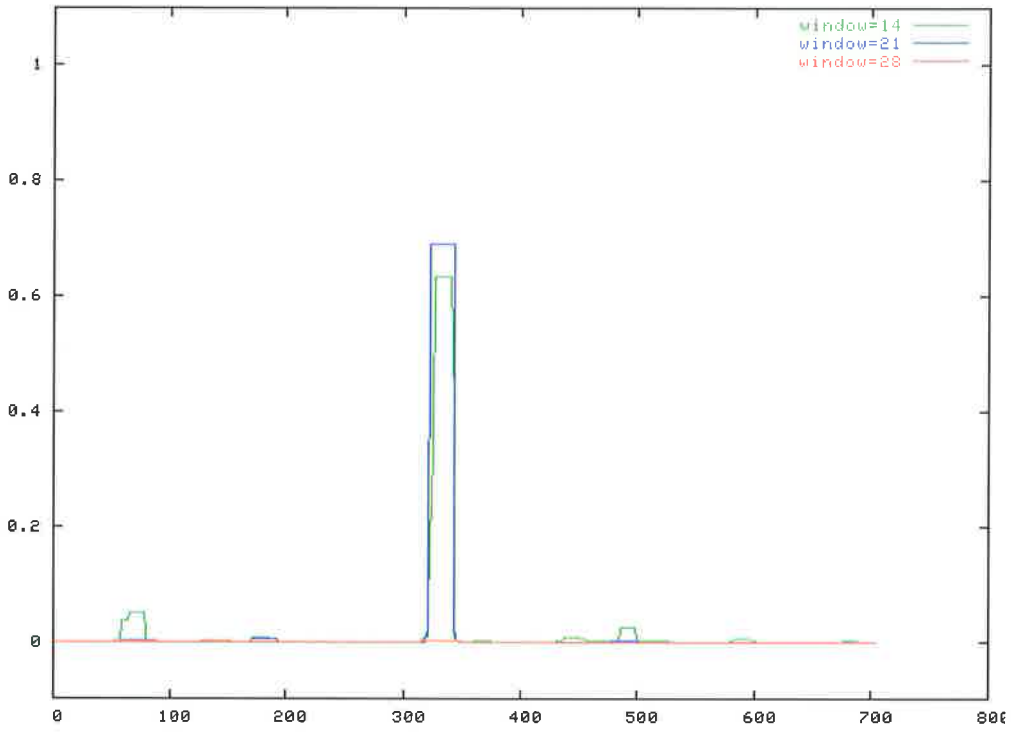


Fig. 3-18 Prediction of coiled coils in the hexamerin sequence from *H. armigera* was performed by Coils software (http://www.ch.embnet.org/software/COILS-form.html). There is one potential coiled-coil site between residues of 300 to 400.

3.2.8 Expression of hemocyanin C and M domains

To study the possible function of hexamerin and to raise antibodies against protein domains, hemocyanin C and M domains from hexamerin were expressed in pQE30 expression vectors, the recombinant proteins were bound to a Ni-column and eluted with guanidine chloride according to materials and methods. The cDNA of the two domains were produced by RT-PCR. Bam HI and Hind III were added to the two ends of the domains. Primers flanked with the restriction sites are listed in Table 2-11. The expected DNA lengths of the C and M domains are 780 and 863 bp in size, respectively. The expected sizes of cDNA from each domain were confirmed on agarose gel electrophoresis, sequencing of the ligated pQE30 plasmid was performed

to examine wether the insert is correct and in reading frame with the expression vector.

Expression of the two hexamerin domains was performed by selecting optimal bacterial growth periods for expression (Fig. 3-19 and 3-21). Once the optimal time was determined, a large-scale protein expression was performed, followed by purification with Ni-beads and dialysis O/N to recover the protein (Fig. 3-20 and 3-22). The expected protein size of the C and M domains was 29.8 kDa and 32.6 kDa respectively (see Table 2-12). Since the vector codes for additional amino acid residues, the actual sizes were larger than the expected size.

3.2.9 Antisera against the two domains of hexamerin

To study the functions of hexamerin, antisera against the recombinant Hemocyanin C and M domains were raised in rabbits according to standard methods described in the method chapter. Final bleeding tests showed that the two sera recognized the bacterial fusion protein (Fig. 3-23) and 85 kDa protein (not shown), therefore, they were used for subsequent functional studies.

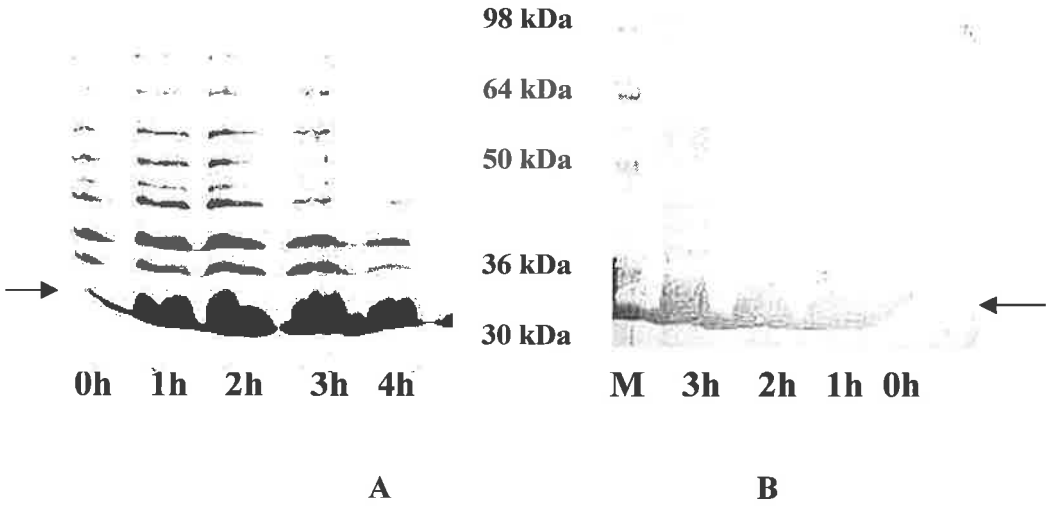


Fig. 3-19 Expressed hemocynain C domain at different time period. SDS-PAGE (A) and Western Blot (B) using anti-poly Histidine serum to analyse the expression of the Hemocyanin-C domain at different time periods. Results showed that at 2h after IPTG application the expression reaches the highest level. The arrows indicate the expressed recombinant proteins.

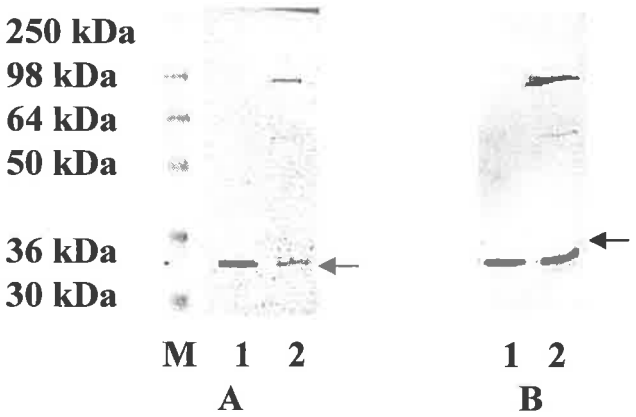


Fig. 3-20 Multimerisation of the purified hemocyanin-C domain. SDS-PAGE (A) and Western Blot (B) using anti-poly-Histidine serum to identify the purified Hemocyanin-C domain under reducing (lane 1) and non-reducing (lane 2) conditions. Under non-reducing condition, the recombinant Hemocyanin domain can form dimers or trimers. The arrows indicate the monomer of the expressed recombinant proteins.

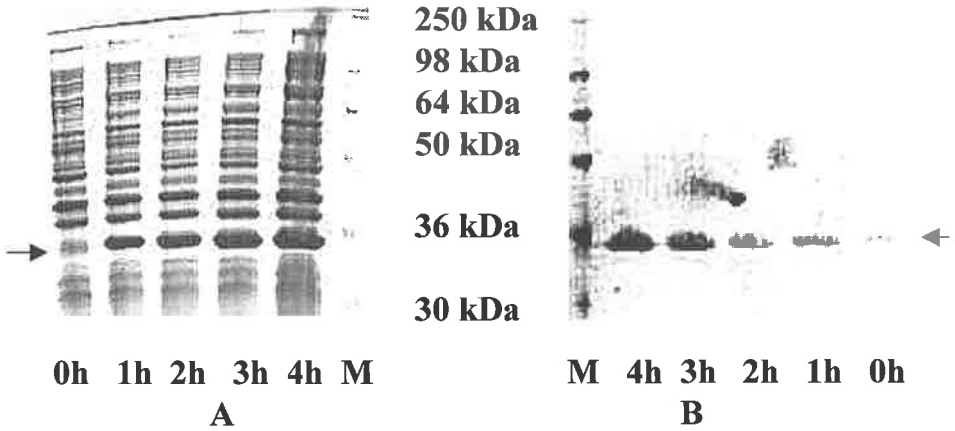


Fig. 3-21 Expressed recombinant hemocyanin-M domain at different bacterial growth periods. SDS-PAGE (A) and Western blot using anti-poly-Histidine serum (B) to analyse the expression of the Hemocyanin-M domain at different bacterial growth periods. The results showed that at 4h after induction the expression reaches the highest level. The arrows indicate the expressed recombinant proteins.

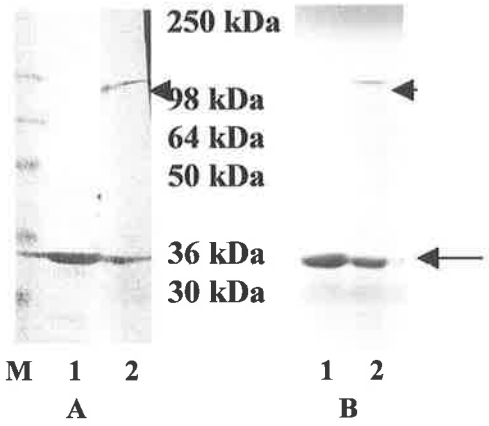


Fig. 3-22 Multimerisaion of the Purified recombinant hemocyanin-M domain under reducing and non-reducing conditions. SDS-PAGE (B) and Western Blot using anti-poly Histidine serum (A) to show the purified hemocyanin-M domain under reducing and non-reducing conditions. Under non-reducing condition, the recombinant hemocyanin M-domain can form dimers or trimers (arrowhead). The arrows indicate the monomer of the expressed recombinant proteins.

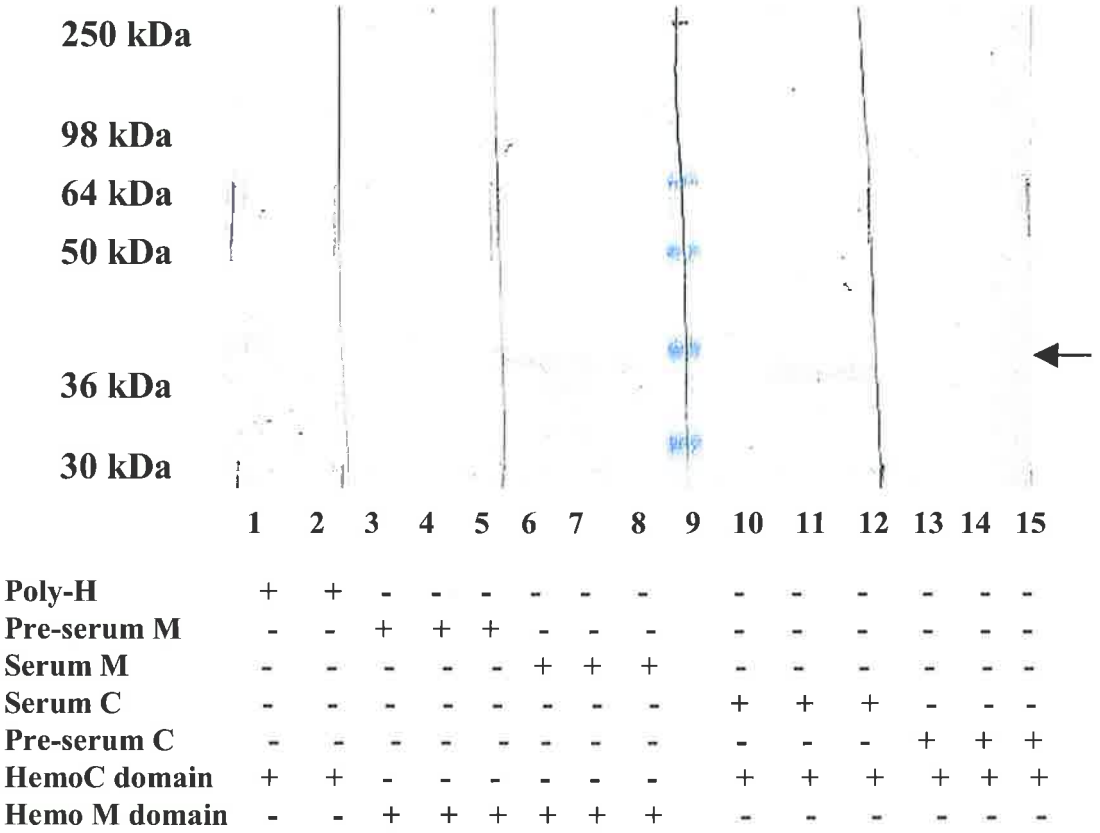


Fig. 3-23 Bleeding test for serum against recombinant hemocyanin C and M domains from *H. armigera*. Lane 5 and 8: 0h for Hemocyanin M domain; Lane 4 & 7: 4h expressed Hemocyanin M; Lane 3 & 6 purified Hemocyanin M domain; Lane 10 &13: 0h for Hemocyanin C domain; Lane1, 11& 14: 4h expressed Hemocyanin M; Lane 2,12 & 15 purified Hemocyanin C domain. Lane 3,4&5, pre-serum against Hemocyanin M; Lane 6,7&8, serum against Hemocyanin M. Lane 10,11&12, serum against Hemocyanin C; Lane 13,14 &15, preserum against Hemocyanin C. Lane1&2 poly-Histidine monoclonal antibody. Arrow indicates the positions of the expressed proteins

3.2.10 Hexamerin is located in the gut

To test whether hexamerin is found in the gut, gut protein extracts were examined on Western blots, using antibodies against recombinant hexamerin. A protein band corresponding to hexamerin in size was stained with the antibodies in extracts from susceptible and resistant larvae (Fig. 3-24). While extracts from susceptible larvae showed a single band, those from resistant larvae showed multiple bands, which could be due to protein degradation or protein modification. It is not known, whether the protein is produced in gut cells or imported from fat body associated with gut. Further experiments are required to determine the origin and the cause of protein heterogeneity in the extracts from resistant larvae.

3.2.11 Different expression of hexamerin is found in eggs

To test the protein expression pattern of hexamerin in the eggs of ANGR and ISOC4 females, Western blots were examined with serum against Hemocyanin M, and GalNAc specific lectin-HPL (see definition of HPL in Table 2-3). It is possible that post-translational modification of hemocyanin by immune-induction could affect hexamerin uptake into eggs. Therefore, hexamerin expression levels and protein patterns in the egg could be different between resistant and susceptible insects. The initial analysis was not conclusive. Surprisingly, staining with anti-hexamerin antibodies did not reveal very strong bands at 85 kDa, but a few proteins between 98 and 250 kDa appeared to be stained and different protein profiles were found between ANGR and ISOC4 (Fig. 3-25). The protein extracts from resistant eggs or females show slightly different hexamerin forms from that of susceptible counterparts (not shown). Furthermore, HPL staining showed that a protein below 250 kDa has a slightly different degree of glycosylation (Fig. 3-25).

Since hexamerin is a storage protein, it may be associated with lipids, which may exist as glycolipids. Further experiments are required to determine the modification of hexamerin.

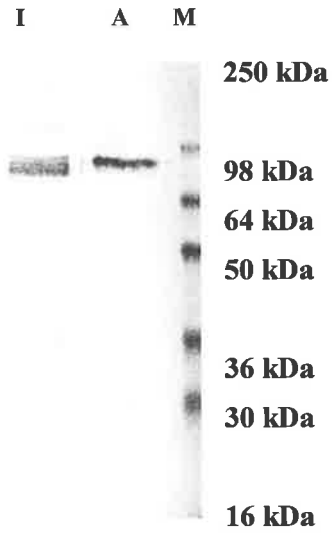


Fig. 3-24 Hexamerin is found in gut. Western blots of gut protein extracts from ISOC4 (I) and ANGR (A) and staining with serum against the hemocyanin C domain from hexamerin after separation on 12% SDS-PAGE. M: Seeblue®Pre-stained standard. Western blot of gut protein extracts using antibodies against recombinant hexamerin as a probe. 20μg of midgut tissue and content from five resistant and susceptible 5th instar larvae respectively.

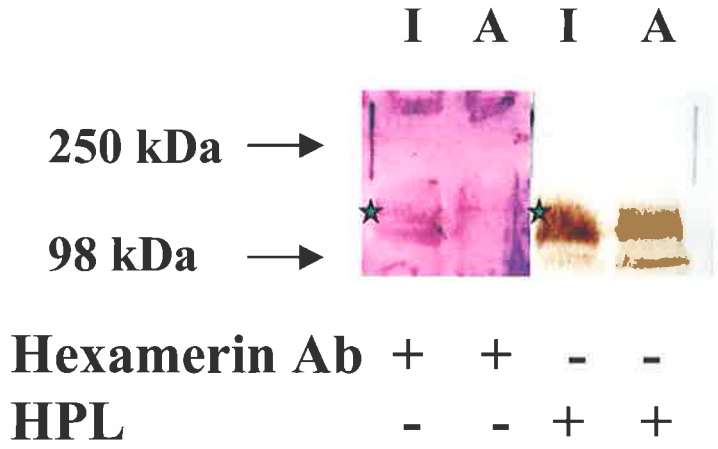


Fig. 3-25 Glycosylation forms of hexamerin in eggs from *H. armigera.* Western blot of egg protein extracts from ANGR and ISOC4 females and stained with anti-serum against hemocyanin C and GalNAc specific lectin-HPL. Egg extracts from ISOC (I) ANGR (A). Both antibodies against Hemocyanin C and HPL showed slight difference (as indicated by stars), which could be due to glycosylation modifications of the target protein. Since the modified proteins are between 98 and 250 kDa in size it is unlikely that the proteins are monomeric hemocyanin. Further experiments are necessary to determine the identity of these proteins.

3.3 Modifications of glycosylation in resistant insects

It is possible that the elevated immune status in resistant insects influences post-translational modifications of proteins. For example, hexamerin secreted into the gut lumen may be glycosylated differently (Fig. 3-24) in resistant insects and thus bind to the toxin thereby inactivating it. Likewise, a modified egg storage protein in resistant females (Fig. 3-25) may trigger immune induction in the embryo, thereby transmitting the elevated immune status to the next generation. A pleiotropic effect of a modifying enzyme, which is affected by the elevated immune response, could therefore explain the phenotypic effects of the ISOC4 strain. Recently, a Bt resistance mechanism, involving a glycosylation modification has been described in nematodes (Griffitts et al., 2001). This mutation inactivates a glycosyltransferase (Gal-T) gene, which causes resistance against Cry5B toxin in *C. elegans*. Therefore, it is possible that alteration of a similar gene in *H. armigera* may contribute to resistance against Cry1Ac in this insect.

3.3.1 Gal-T antibodies cross-react to *H. armigera* protein

Protein extracts from ANGR and ISOC4 *H. armigera* fat body were tested on Western blots using two different batches of antisera against *C. elegans* Gal-T (Appendix 4).

When Western blots containing fat body proteins were incubated with one of two batches of antiserum (92-1) against *C. elegans* Gal-T, a protein band of ca. 40 kDa in size was stained (Fig. 3-26). In addition to this protein, a number of other proteins between 40 and 150 kDa in size were also stained (Fig. 3-26). While the 40 kDa protein staining was much stronger in the ANGR than in the ISOC4 strain, the other

cross-reacting bands where similar between the two strains. This suggested that antiserum against a nematode Gal-T cross reacts with a *H. armigera* protein of similar size, which is differently expressed in the two strains.

Fig. 3-26 Gly-T antibodies cross-react with BBMV preparations from *H. armigera.* Western blot shows the competition between HPL and Cry1Ac for binding fat body protein activity. Left panel showed that the blot only stained with HPL at 0.02%(V/V) in PBS O/N, whereas on right panel, Blot first pre-stained 2h with Cry1Ac (0.01%) in PBS then HPL stain O/N. After HPL developing, right panel (arrow head) indicated that a band ca.98 kDa was lost suggested that protein was bound by Cry1Ac.Then Blots were further stained with serum against Gly-T from *C. elegans*. Significant difference occurred with respect to a band ca. 40 kDa (arrow), that had a similar size to Gly-T from *C elegans* in size was discovered. A: ANGR, I: ISOC4.

3.3.2 Gal-T activity in the two *H. armigera* strains

To test whether there was a difference in the Gal-T activity between the two strains, direct measurements of Gal-T activity from brush border membrane vesicles (BBMVs) was performed. The results showed a significant difference between ANGR and ISOC4 where using an enzyme-linked lectin assay (ELLA). ANGR had a significantly higher activity than ISOC4 (Fig. 3-27). Since the Gal-T activity differed

between the two strains, which may affect hexamerin and Bt-receptors, gut-derived glycoproteins were further studied.

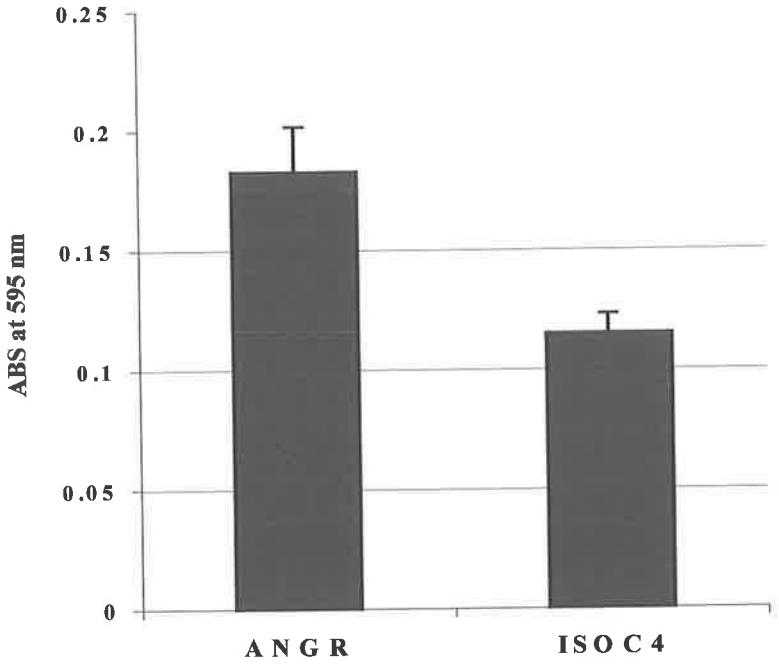


Fig. 3-27 Gly-T activity of BBMVs measured by using the ELLA method. Gly-T activity of BBMVs from ANGR and ISOC4, measured by using the ELLA method. Gly-T activity of ANGR was significantly higher than that of ISOC4 even though some variations are observed. The error bars indicate 99% confidence intervals and show mean + SD (n=3).

3.3.3 Induction of hexamerin RNA

To examine whether induction of hexamerin is regulated differently in the two strains, both ISOC4 and ANGR larvae fed with sub-lethal doses of Cry1Ac and RNA expression analysed on Northern blots. Induction of RNA was visible in both strains with similar amounts of RNA produced (Fig. 3-28). This suggests that hexamerin is not constitutively induced in ISOC4 larvae, but is induced after gut-derived elicitor application. Since hexamerin expression in larvae is low compared to adult females, it remains to be seen whether hexamerin expression is increased in immune-induced females and in eggs.

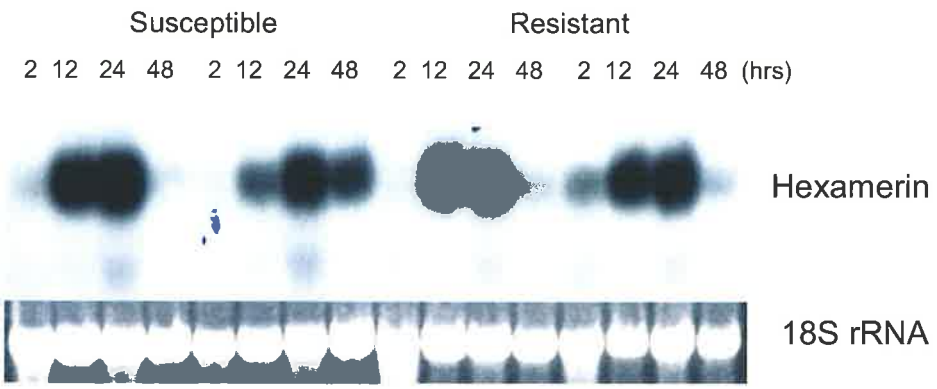


Fig. 3-28 Induction of hexamerin gene in *H. armigera* fat body. Maximal expression occurs between 12 and 24 hours after induction.

3.4 Is a membrane-bound alkaline phosphatase a Bt-receptor?

When the second batch of antiserum (93-1) against nematode Gal-T was used to incubate protein extracts of BBMVs from the two strains, staining of a protein at 82 kDa was observed (Fig. 3-29). When negative controls of these Western blots were analysed, the staining remained even in the absence of first and second antibodies (Fig. 3-29). The staining disappeared when peroxidase conjugated secondary antibodies were used (not shown). This suggests the staining was not due to specific binding of antibodies to the 82 kDa protein, but due to a possible alkaline phosphatase activity of the p82 protein. Surprisingly, by coincidence, the 82 kDa protein was stained differently in ANGR and ISOC4 BBMV extracts. This suggests that a membrane-bound alkaline phosphatase from BBMVs may be altered in the resistant insect strain.

3.4.1 Characterisation of an alkaline phosphatase from *H. armigera*

3.4.1.1 BBMV preparation and SDS-PAGE, in-gel digestion, and HPLC

Based on the above results, alkaline phosphatase from *H. armigera* was further characterised. This was done by sequencing the corresponding protein from the BBMV protein extracts (Fig.3-30). The 82kDa protein band was cut from the gel and digested by placing the gel pieces in trypsin and digestion buffer. The polypeptide extracts were separated by RP-HPLC (Fig. 3-31), and individual protein peaks collected and sequenced. Ten samples were collected manually and four of them were sequenced by a protein sequencer according to the method of Edman and Begg (Edman and Begg, 1967).

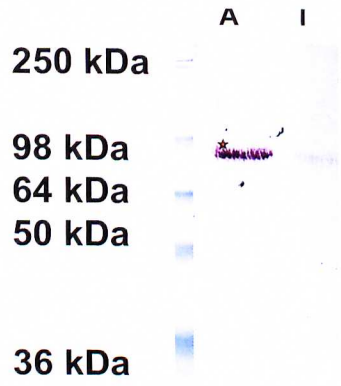


Fig. 3-29 The ALP activity of BBMVs from ANGR and ISOC4. 20 μg of BBMV was separated by 12% SDS-PAGE. The gel was run under non-reducing conditions. The protein was then transferred to NC. The blot was first washed with PBST for 5 min, then, developed in ALP substrates of NBT and BCIP. Star indicated the ALP activity from ANGR BBMV extract

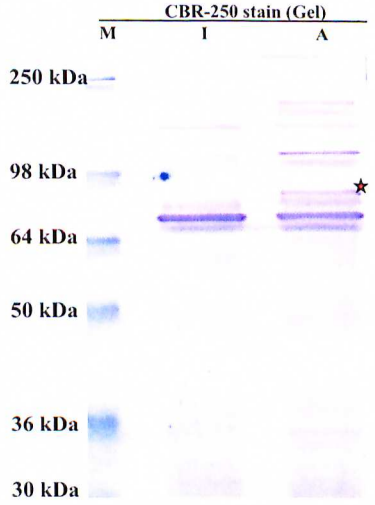


Fig. 3-30 Comassie blue staining of BBMVs from ANGR and ISOC4 under non-reducing conditions. The BBMV preparations from ANGR (A) and ISOC4 (I) were separated on 12% SDS-PAGE under non-reducing conditions and stained with Comassie Brilliant Blue. Star indicates the p82 protein in ANGR BBMV extracts.

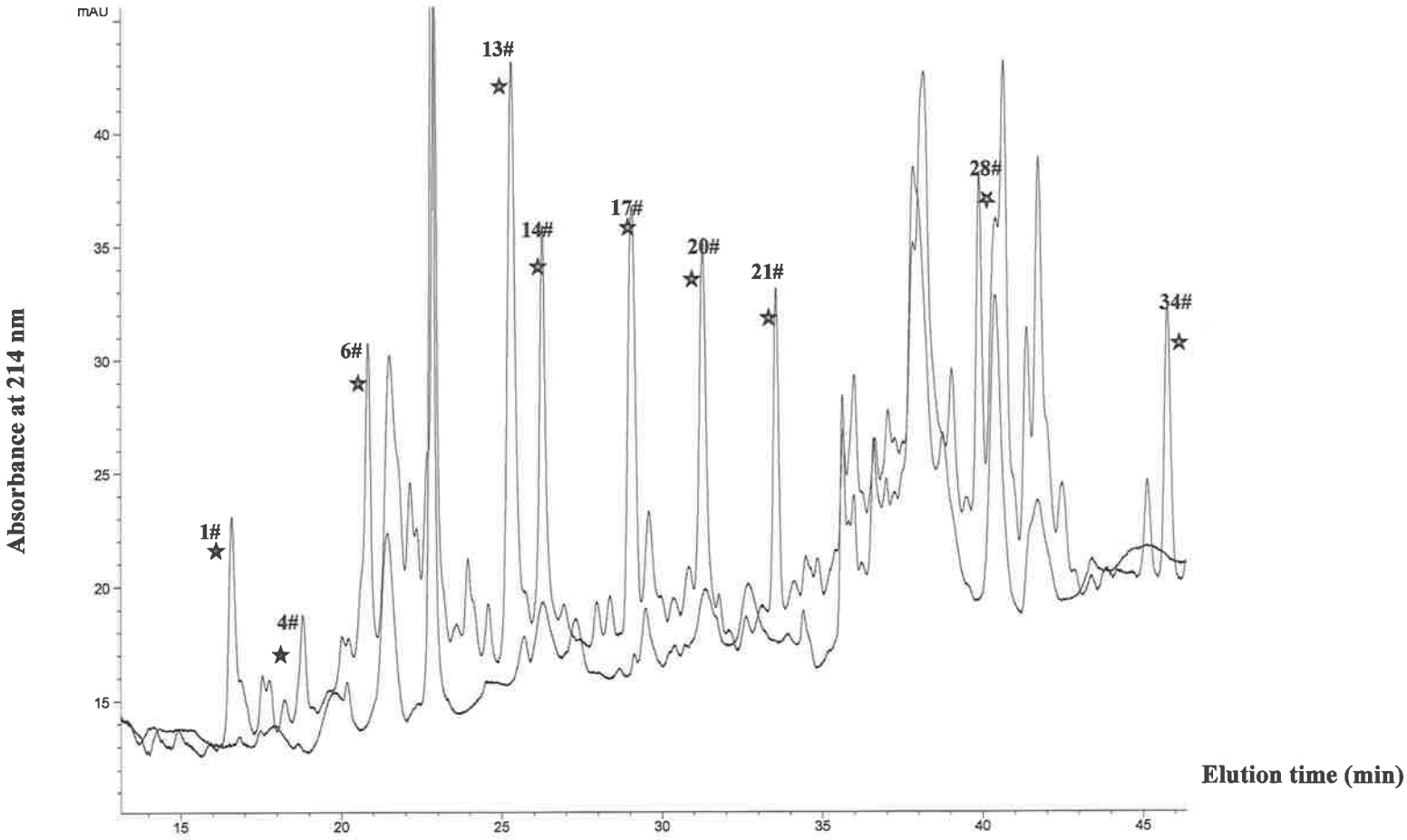


Fig. 3-31 RP-HPLC of the trpsin-digested p82 protein from ANGR BBMVs. Separation of polypeptides extracts after RP-HPLC, several specific peptides (indicted by the stars) were collected manually. Peptides 14, 17, 21 and 34 were used for further peptide sequencing.

3.4.1.2 Peptide sequences

Four peptide sequences, which were collected from the RP-HPLC fractions, were used for peptide sequencing. The corresponding amino acid sequences of the four peptides are listed in Table 3-3.

Table 3-3 Four peptide sequences from ALP of *H. armigera*

Fraction No.	Peptide sequence
No. 34	IDHAHHDNLVXALD
No. 21	AETSANYWAQDADAA
No. 17	
Major:	AFLPNTVVDDMSYG
Minor:	TWENDGEQSQE
No. 14	DXPDIAHQLVHHHPG

3.4.1.3 Blastp analysis

The sequences of the four peptides were compared to other proteins in the data bank by Blastp analysis using an almost exact match program. The four peptides showed high similarity to the membrane-bound alkaline phosphatase (ALP) sequence from *B. mori*. Their potential positions in the corresponding protein sequence are listed in Fig. 3-32.

This suggests that a protein from BBMVs with alkaline phosphatase activity is related to a *B. mori* protein, which exists in a soluble and membrane-bound form. Since alkaline phosphatases have been suggested to play a role as receptors for the Bt toxin (Jurat Fuentes and Adang, 2004), these findings indicate a possible role for ALP in Bt

resistance in *H. armigera*. The difference in expression of ALP could be due to a mutation in the ALP gene of the ISOC4 strain or indirect changes where the ALP protein may be another substrate for a glycosylation modification enzyme, which is different in the resistant strain. Further experiments are required to clarify the reasons why the 82 kDa protein is down-regulated in the ISOC4 strain.

21# AETSANYWAQDAQAA

MVVSCGGGGGGGGAGEGRGPLPPGAARAGEASAATRSA**AESEASFWVREAQEA**IETREREGAGAKQ

AAGHAKNVVMFLGDGMSVPTLAAARTLLGQRRGQTGEEASLHFEQFPTLGLAKTYCVNAQVPDSSC

TATAYLCGVKANQGTPGVTAAVPRHDCEASTDVTKRVQSIAEWALADGRDVGIVTTTRITHASPAG

17# TWENDGEQSQE 14#DXPDIAHQLVHHHPG 17# AFLPNTVVDDMGSYG

TFAKVANR**NWENDNDVKQE**GHDVN**RCPDIAHQLIKMAPG**NKFKVIFGGGRR**EFLPTTQVDEEGTRG**

LRTDGRNLIEEWQNDKESQKVSYKYLWNRQELLKLGSSPPDYLLGLFEGSHLQYHLEGDESTEPTL

34# IDHAHHDNLVXLALD

AELTDVAIRVLSRNERGFFLFVEGGR**IDHAHHDN**YAH**LALD**ETIEMDRAVKVATDALKEDESLVVV

TADHTHVMSFNGYSPRGTDVLGTVRSLDSNRMPFMVLSYANGPGARIQQNGVRPDVTTDANFGALR

WRTHTDVPLDSETHGGDDVTVFAWGVHHWMFSGLYEQTHVPHRMAWAACMGPGRHVCVSAATVPTA

ALLSLLLAAFITLRHQCFL

Fig. 3-32 The putative position of the four peptides and a minor peptide from ALP of *H. armigera* in the ALP sequence of *B. mori*.

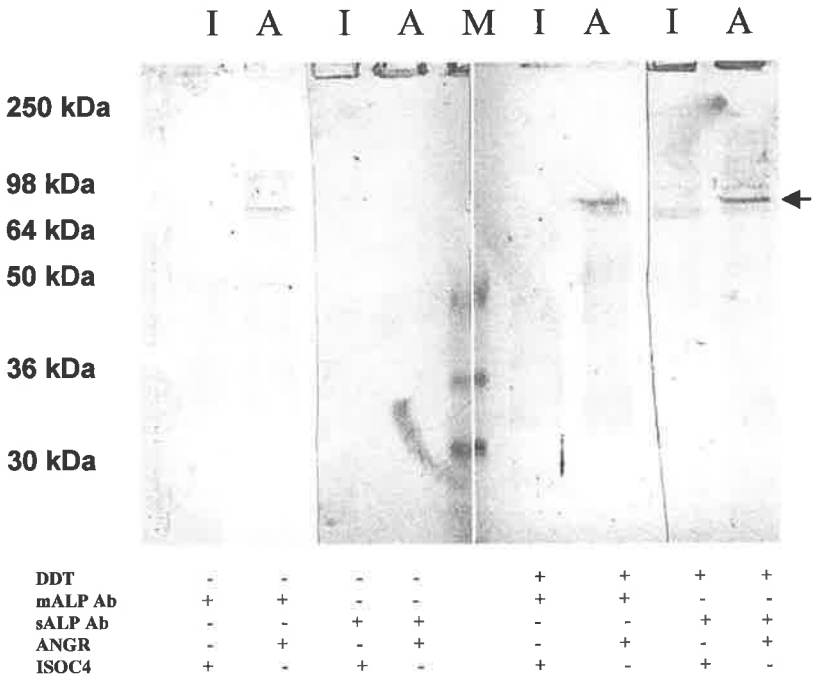


Fig. 3-33 BBMV preparations were tested with ALP antibodies from *B. mori*. Western blots of BBMV preparations of ANGR (A) and ISOC4 (I) were run under both reducing and non-reducing conditions, and subsequently stained with both mALP and sALP antibodies from *B. mori*. The secondary antidody was conjugated with alkaline phosphatase conjugate. Arrow indicates the position of ALP.

3.4.2 Western blots stained with serum against membrane bound ALP or soluble ALP from *B. mori*

To explore whether ALP is altered in the Bt resistant strain, the serum against both membrane-bound ALP (mALP) and soluble ALP (sALP) from *B. mori* (Appendix 4). Western blots stained with both antibodies showed that there were significant differences between protein extracts from ISOC4 and ANGR BBMVs. It was interesting to note that under non-reducing conditions, the blot did not stain with antibodies against the soluble alkaline phosphatase (sALP), but stained with antibodies against the mALP (Fig. 3-33).

3.4.3 Cry1Ac binds to ALP, which is ConA specific

To determine whether ALP is the receptor of Cry1Ac or not, Cry1Ac binding to BBMV preparations of both ANGR and ISOC4 was performed using the qualitative binding method of (Sarah et al., 1999). To check the glyco-determinants on ALP, the gut extract blot was also stained with ConA (a glucose-specific lectin). The results suggested that Cry1Ac binds to ALP, which probably contains a ConA specific sugar residue (Fig. 3-34 and Fig. 3-36). Blots stained with HPL and PNA revealed that these two lectins did not bind to ALP (Fig. 3-35).

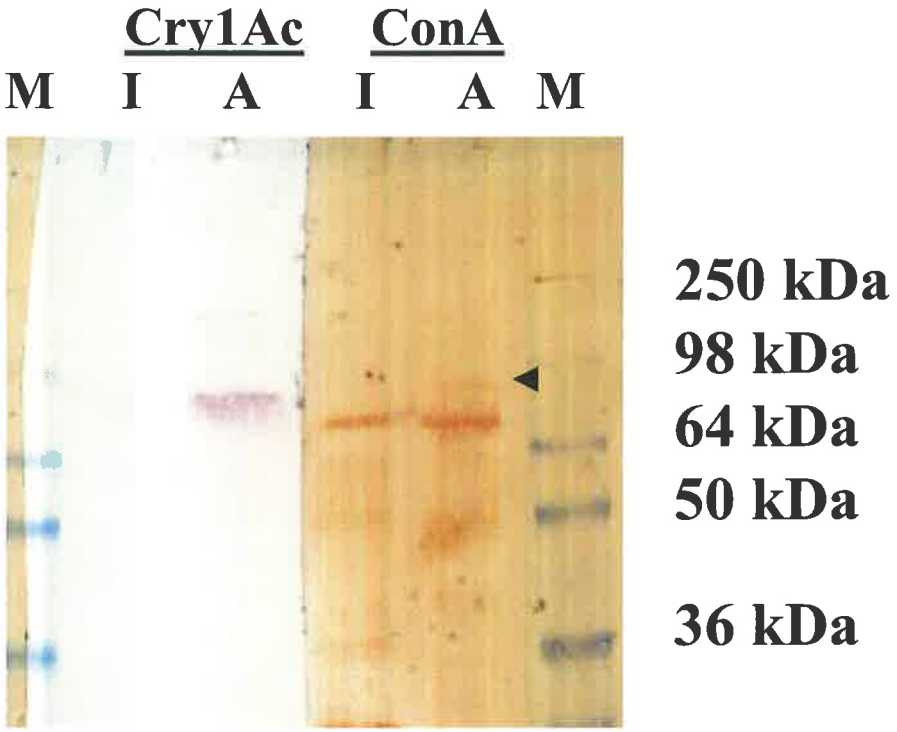


Fig. 3-34 Cry1Ac and ConA staining of BBMVs from ANGR (A) and ISOC4 (I). The ALP protein specifically binds to Cry1Ac and weakly to ConA (arrow indicated). Note the ConA stained band at about 70 kDa, which is equally strong in protein extracts from both strains.

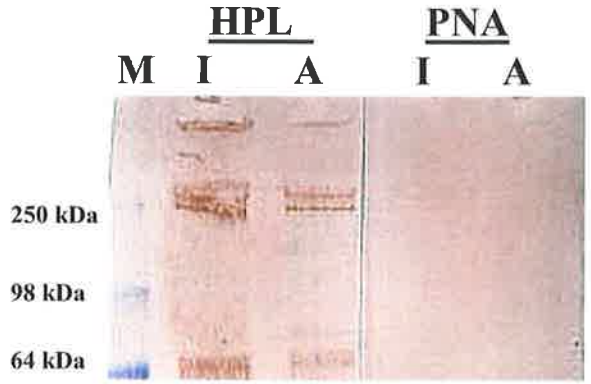


Fig. 3-35 HPL and PNA staining of BBMV preparations. Western blot of BBMV extracts from ANGR (A) and ISOC4 (I) under non-reducing conditions, HPL, and PNA stain. M: SeeBlue® protein marker. There is no visible cross reaction with either PNA or HPL lectin in the 82 kDa region.

Fig. 3-36 Lectin staining of BBMV preparations from *H. armigera.* Western blot of the BBMV preparations of ANGR (A) and ISOC4 (I) stained with peroxidase conjugate lectins, HPL, ECL, Jacalin, SBA, WBA, and Con A. The blot was exposed to X-ray film and developed. The star indicates the 82 kDa protein of ALP, which is a Con A containing glycoprotein

Chapter 4 Discussion

Reports of the emergence of resistance to Bt in field populations of DBM have highlighted the inherent danger of the evolution of resistance against this powerful insecticide (Ferre and Van Rie, 2002). To avoid or delay Bt resistance, several Bt resistance mechanisms were proposed. The most effective and best-characterised resistance mechanisms are based on receptor-inactivation at the midgut membrane, for example involving the aminopeptidase N (Rajagopal et al., 2002) and the cadherin-like (Gahan et al., 2001; Morin et al., 2003) gene families. Other mechanisms known to impair Bt-toxicity include alterations to proteolytic activity of midgut extracts affecting pro-toxin processing and maturation (Oppert, 1999), and increased rates of replacement of damaged cells at the gut lining by stem cells (Martinez-ramirez et al., 1999).

4.1 Bt resistance in *H. armigera*

While most resistance mechanisms in field or laboratory selected populations are recessive and based on receptor inactivation, there are occasional reports of dominant or semi-dominant mechanisms of resistance or tolerance with unusual characteristics. For example, Bt-resistance was selected in a *H. armigera* population from the field by feeding the toxin with artificial diet (Akhurst et al., 2003). The resulting resistance was based on a single semi-dominant mutation. This population was back-crossed with a susceptible strain to get nearly isogenic resistant and susceptible lines. After four crosses and selection for resistance, the resulting ISOC4 strain was analysed for Cry1Ac-binding to BBMVs. This assay showed differences in binding to an unknown

protein, while none of the known aminopeptidase N genes showed any mutation (Angelucci et al., 2002). Moreover, exposure to Bt toxin imposed a fitness cost, which is likely to derive from pleiotropic effects of the resistance gene rather than effects from other gene loci (Bird and Akhurst, 2004). This suggested that resistance was not based on the inactivation of one of the major receptor genes but on indirect effects from another unknown gene, which also affected developmental and immune functions.

4.2 Immune status in ISOC4

Insect immune responses to pathogens or its related toxic products, such as Bt cry toxins, are affected by physiological and ecological factors: fitness costs, life history and the genetics of resistance (Rolff and Siva-Jothy, 2003). For example, strong gut blackening in resistant ISOC4 larvae clearly indicates that Bt resistance might be associated with elevated melanisation. On the other hand, the elevated immune status results in fitness costs, such as delayed development (Bird and Akhurst, 2004). Moreover, the genetics of Bt resistance was affected by selection pressure. It is a semi-domnant trait when the toxin concentration is high, such as in exposure to transgenic plants expressing active toxin. However, when crystal were mixed with food, the actual amount of active toxin may be much lower, which would exposure larvae to low dosage of toxin. Under theses conditions, the resistance may be expressed as dominant trait and show a matenal effect. Further studies found that dominant traits may be associated with Bt resistance in ISOC4, which might be due to pleiotropic effects of a single gene. Glycosylation plays an important biological role for hosts in recognition of self and non-self. To keep pace with pathogen, the host will use diverse forms of oligosaccharides to avoid the invasion of pathogens or its

toxic product (for example, Bt toxins), while maintaining its functions (Gagneux and Varki, 1999). In the current project, Gal-T was shown to be changed and may contribute Bt resistance.

4.2.1 Melanization and coagulation

Morphological inspection of the ISOC4 strain showed a melanization reaction visible in the gut lumen, which may be associated with an elevated immune status in the resistant strain (Fig. 1-4). Further analysis showed that melanization in cell-free hemolymph (plasma) and gut was significantly higher in the ISOC4 than in the susceptible ANGR strain (Fig. 3-1). This is associated with a higher coagulation reaction in hemolymph from the ISOC4 strain, which precluded protein analysis using biochemical approaches.

Coagulation is an important step in wound healing and immune response for insects and vertebrates (Li et al., 2002; Scherfer et al., 2004). Coagulation is achieved by the aggregation of proteins in the presence of elicitors or adhesion molecules. The identification of coagulating proteins in different arthropod species suggests that a diverse group of proteins serve as pro-coagulants (Theopold et al., 2002; Theopold et al., 2004) that include lipophorin (Duvic and Brehelin, 1998; Scherfer et al., 2004), hexamerin (Ma et al., 2005; Scherfer et al., 2004) and vitellogenin (Hall et al., 1999). For example, lipophorin is a major lipid carrier, but has also been associated with coagulation reactions (Li et al., 2002; Ma et al., 2005). Likewise, the role of hexamerin and vitellogenin as egg storage proteins are well characterised, but their role in coagulation is not well known. It is known that pro-coagulants are cross-linked into long fibres in the presence of transglutaminase (Hall et al., 1999). The fibres are

involved in forming clots and a cross-linked mesh-work of cells and plasma to seal off wounds and trap micro-organisms (Nardi et al., 2005; Theopold et al., 2004).

In addition to reactions leading to fibrous clots, another less well defined coagulation reaction occurs inside the hemocoel, presumably in the absence of oxygen, and involves the formation of globules less than one tenth of the size of a cell (Nardi et al., 2005; Theopold et al., 2004). The glycoproteins contributing to globule formation are stored in granules of hemocytes and are released into the hemolymph upon immune activation. One possible mechanism of globule formation is the interaction of Gal-containing pro-coagulant glycoproteins released into the hemolymph, where they interact with the tetrameric Gal-specific lectin, that circulates in the hemolymph (Castro et al., 1987). This suggests that some forms of coagulation reactions are dependent on glycosylation (Korayem et al., 2004).

4.2.2 Hexamerin induction and altered gut glycosylation

To study immune induction and the effects of an elevated immune status on Bt resistance, a gut-derived immune induction reaction was developed by feeding sub-lethal doses of the Bt toxin Cry1Ac with the artificial diet. Since this method has not been used before, it is not clear exactly what causes the immune induction. Given that *B. thurigiensis* bacterial extracts without the toxin do not elicit an immune response, it is likely that the crystal toxin causes some damage to the gut lining, which then brings toxin molecules or contaminating bacterial elicitors in contact with the hemolymph. Under these conditions the effects of the immune induction could be studied in hemolymph from ANGR larvae. It was found that the gut-derived Bt elicitor induced an 85 kDa hemolymph protein. This protein was previously identified as a possible Cry1Ac-binding protein, which appeared to differ in amount in resistant

and susceptible larvae (Nicki Featherstone, Per. Comm.). However, the amount of protein in non-induced larvae was insufficient to obtain protein sequence.

To determine the identity of p85, PCR cloning was performed using an in-gel protein digest, peptide sequence analysis and PCR-primer determination using extracts from induced larvae. The coding DNA was sequenced and showed strong similarity to hexamerin from *H. virescens.* Antibodies created against a recombinant peptide from the *H. armigera* hexamerin were obtained in rabbits and cross-reacted with p85. Using the anti-hexamerin antibodies it was also established that hexamerin is found in the gut lumen in addition to the hemolymph. In protein extracts from ISOC4 gut preparations, multiple bands of hexamerin are detected, which may be caused by proteolytic digestion or post-translational modifications. Since most other proteins from the gut of ISOC4 are not dramatically changed, it is unlikely that the multiple bands seen in the hexamerin molecule are derived from over-digestion of gut proteins. However, overexpression of a specific protease in the gut of ISOC4 larvae is not ruled out, given that immune induction may be responsible for changes in specific gut proteases resulting in resistance (Li et al., 2005).

An alternative explanation for multiple hexamerin bands in ISOC4 extracts is post-translational modifications. Altered glycosylation due to a reduction of Gly-T has been demonstrated in nematodes (Griffitts et al., 2001) and a similar process may occur in insects. However, recent observations in nematodes and insects suggest that the glycosylation changes in resistant animals affect glycolipids (Griffitts et al., 2005) rather than glycoproteins. This could explain why the hexamerin protein was not affected by alterations in the mutant strain. If hexamerin is associated with lipids, a

functional change in the lipoprotein could affect its pro-coagulant properties. Changes in glycosylation may also affect the production of glycoproteins, causing a reduction of protein secretion. Since the combined amounts of hexamerin protein bands in ISOC4 exceeds that of the protein band in ANGR, it is likely that protein secretion is not negatively affected. Since hexamerin is immune-induced and a pro-coagulant (Scherfer et al., 2004), it is possible that the function of the protein particle, which may be comprised of six monomers and lipids, is altered in ISOC4. The protein is secreted into the gut of wild-type larvae performing an unknown function. This function may be similar to lipophorin, which is secreted into the gut lumen where it functions as a lipid carrier (Hall et al., 1999; Ryan and van der Horst, 2000). Since hexamerin is also a pro-coagulant in the hemolymph, both hexamerin and lipophorin could perform dual functions in the gut lumen. While apolipophorin I interacts with LPS (Appendix 1.2), hexamerin appears to interact with oligomeric GalNAc-containing lectins including Cry1Ac.

As indicated above, pro-coagulants in the hemolymph depend on specific glycosylation modifications. For example, Gal-containing glycoproteins are absent in the circulating plasma, but are released from hemocyte granules upon immune stimulation to form aggregates with Gal-specific lectins circulating in the hemolymph (Castro et al., 1987; Korayem et al., 2004). If pro-coagulants are secreted into the gut lumen of immune-induced insects, the glycosylation of the pro-coagulant may resemble pro-coagulants in granules. In this scenario, the activated pro-coagulant, such as a modified hexamerin, may interact with gut-derived lectins, such as mature Bt-toxins. Further experiments, involving the exact glycosylation status of hexamerin or its associated lipids in ISOC4 and ANGR gut extracts are required to reveal its

function in Bt resistance. If hexamerin in the gut forms aggregates with Bt-toxin as in hemolymph it may prevent the toxin from reaching the gut lining. Since the mature toxin was shown to be oligomeric, which is a requirement for the formation of aggregates, this suggests that the active form of the toxin is probably oligomeric. Finally, if sugar-specific glycosylation is altered as a result of immune induction, the target proteins may not be restricted to pro-coagulants but include other proteins, such as possible receptors on the BBM, which are known to have sugar-specific O-glycosylation (Burton et al., 1999).

Post-translational modifications of receptors and pro-coagulants may be compatible with previously reported observations (Akhurst et al., 2003; Liao et al., 2002), including changes in Cry1Ac-binding to an unknown protein of about 82 kDa (Angelucci et al., 2002), and possible interactions with pro-coagulants reported in this work. Such pleiotropic effects are consistent with a model of resistance or tolerance to the toxin based on inducible gene functions that affect post-translational modification of multiple proteins, rather than rare mutations in a receptor gene.

4.2.3 Gly-T and Bt resistance in *H. armigera*

Since hexamerin or associated glycolipids in the ISOC4 strain may be altered by post-translational modifications, it is possible that glycosylation enzymes are different in the resistant strain either directly or indirectly as a result of the elevated immune status in ISOC4. Therefore anti-glycosyltransferase antibodies from nematodes (Griffitts et al., 2001) were tested for cross-reactivity to insect glycosyltransferases. In Western blots from gut extracts a number of protein bands were found to cross-react with the nematode antibodies. One band at about 40 kDa was the strongest cross-reacting protein and staining to this band was weaker in ISOC4 extracts compared to

ANGR extracts. Moreover, Gly-T enzyme activity in BBMVs from the gut was found to be different in ISOC4 and ANGR. Since Gly-T can transfer sugars onto proteins or lipids (Griffitts et al., 2005), it may modify hexamerin or its associated lipids thereby altering the affinity to the toxin in the gut lumen, which would be a dominant trait. The observation that the resistant *H. armigera* had a reduced Gly-T activity is no proof, but compatible with a change in post-translational modifications of gut proteins in the ISOC4 strain.

The binding of GalNAc-specific oligomeric lectins to induced hexamerin in plasma suggests that glycosylation may be a prerequisite for the observed aggregation reaction. Although there are other mechanisms (Banks et al., 2001), crystal toxins may use sugars to bind to aminopeptidase receptors at the brush border membrane (Burton et al., 1999; Derbyshire et al., 2001). This opens up a number of scenarios for toxin interactions in the gut lumen. Hexamerin, like other immune proteins, may be secreted into the gut lumen in specific glycosylated form that interacts with the mature toxin to form insoluble aggregates. Interestingly, sugar modifications that cause coagulation in hemolymph plasma appear to be different in the gut lumen, where Gal-specific lectins co-locate more strongly with toxin-binding globules (Sarjan, 2002), whereas GalNAc lectins are bound to the peritrophic membrane. In hemolymph, Gal-containing glycoproteins with pro-coagulant activities are mainly found inside hemocyte granules rather than in circulating plasma (Korayem et al., 2004; Scherfer et al., 2004). As a working hypothesis, it is possible that glycoforms of pro-coagulants secreted into the gut lumen of immune-activated insects resemble pro-coagulants stored inside granules of hemocytes. The presence of immune-active

pro-coagulants would allow interaction with sugar-binding proteins from pathogens and their toxins forming a first line of defence in the gut lumen.

4.2.4 ALP and Bt resistance in *H. armigera*

When Western blots of gut protein extracts were developed with alkaline phosphatase reagents, a stained band was detected in ANGR extracts, which was significantly reduced in ISOC4 extracts. This chance observation suggests that a gut alkaline phosphatase retains enzymatic activity after SDS-PAGE and this activity is significantly reduced in the resistant strain. Microsequencing of this band produced four peptides with sequences similar to a membrane-bound *Bombyx mori* alkaline phosphatase. Further experiments will show whether the observed changes in ISOC4 are due to post-translational modifications, or genetic alterations of the alkaline phosphatase gene. Since membrane-bound alkaline phosphatase on BBMVs are known to bind to Cry1Ac, a genetic mutation within the ALP gene in the ISOC4 strain may account for the recessive trait of Bt resistance at high dosage, which did not show a maternal effect (Bird and Akhurst, 2004). Alternatively, the changes in the ALP protein may be due to indirect effects, such as post-translational modification, reducing the amount of secreted protein.

In *H. virescens*, ALP is a GalNAc containing glycoprotein and a Cry1Ac toxin receptor (Jurat-Fuentes and Adang, 2004). Here it is shown that ALP from *H. armigera* also binds Cry1Ac (see Fig. 3-33, Cry1Ac binds to BBMVs from *H. armigera*). This ALP is also glycosylated, but it is ConA-binding, which indicates that it is a glucose or mannose-containing glycoprotein. No significant GalNAc glycosylation of ALP was observed. If ALP is a receptor for Cry1Ac, it is probably not based on Gal or GalNac-specific binding, as suggested for aminopeptidase N.

Alteratively, if these two sugar epitopes were involved in Cry1Ac-binding to ALP, a mannose isomerase may be involved in Bt resistance as in *P. xylostella,* where resistant insects have stronger mannose isomerase activity (Herrero et al., 2001).

In *H. virescens* the resistant line, YHD2, showed altered glycosylation of ALP, which was suspected to be involved in Bt resistance (Jurat-Fuentes and Adang, 2004). Here, in *H. armigera*, mALP was shown to be a potential Cry1Ac receptor based on reduced ALP activity observed in resistant compared to the susceptible insect.

It was previously found that loss of binding sites in the BBMVs from the resistant strain of *H. armigera* were correlated with Bt (Cry1Ac) resistance (Akhurst et al., 2003). Further characterization of the Cry1Ac-binding proteins from BBMVs identified five proteins on SDS-PAGE (Angelucci et al., 2002). Four of these were identified as APNs and showed no difference between resistant and susceptible strains that could be responsible for the difference in toxin binding. This suggested that APNs did not play an important role in Bt-resistance in the ISOC4 strain. However, one protein of about 82 kDa in size, which could not be characterized due to ambiguous mass spectrometry data (R. Akhurst, Pers. Comm.), was shown to bind Bt. This uncharacterised protein may be important for expression of Bt resistance in the ISOC4 strain of *H. armigera* (Angelucci et al., 2002). The chance observation that a membrane-bound alkaline phosphatase of a similar size to the unknown protein (Angelucci et al., 2002) exists in *H. armigera* and binds Bt suggests that mALP may be a Cry1Ac receptor and altered in the resistant strain ISOC4 of *H. armigera*. Since phosphorylation of proteins is an important pathway in immunity, alterations of ALP proteins may also cause (partial) induction of immune reactions. In this speculative

scenario, the altered ALP may cause pleiotropic effects, including the loss of toxin-binding and partial activation of the immune system.

4.2.5 Maternal-effect and Bt resistance in *H. armigera*

If the tolerance to Bt toxin is caused by a transient induction of the immune system, which is restricted to larval and adult stages but not affecting embryonic functions, the effect should not be visible in the next generation. However, since hexamerin is a storage protein and incorporated into oocytes during oogenesis, it is conceivable for a modified hexamerin to induce the immune system in the embryo, or directly sequester the toxin in the embryonic gut, where it may protect the emerging neonates against the toxin. If this is correct, then neonates will only be protected from Bt by the immune response if they derive from a resistant mother, whereas the offspring from susceptible mothers will be susceptible. To test this assumption, reciprocal crosses were made and bioassays performed on the progeny. In these experiments a component of the Bt tolerance in ISOC4 was transmitted via a maternal effect when *H. armigera* larvae were subjected to relatively low doses of Cry1Ac toxin in the artificial diet. When resistant females were mated with susceptible males, the offspring showed a higher tolerance to Cry1Ac compared to offspring from the reciprocal cross. Furthermore, the tolerance was not sex-linked. However, the results from the two reciprocal crosses also showed that embryonic-encoded traits also contributed to Bt resistance.

The nature of the functional protein in the ovary that leads to maternal transmission of the immune status is not known. Since hexamerin is an egg storage protein (Terwillinger, 1999) that is transported from the hemolymph into the ooplasm during vitellogenesis (Burmester et al., 1998), a modified form of hexamerin or its associated

lipids is a potential candidate. However, further experiments are needed to identify the elicitor and its role in the transmission of tolerance to the toxin. Similar experiments with reciprocal crosses of ISOC4 and ANGR using Cry1Ac from transgenic plants did not reveal a maternal effect (Bird and Akhurst, 2004). The reasons for this apparent difference are not known, but a possible reason for the difference could be the relative age of neonates used for the bioassay of the genetic crosses. If maternal effects are based on gut-derived pro-coagulants, the effect may be short-lived. Another reason may be the application of Cry1Ac via transgenic plants versus protoxin in the diet. Since the toxin in transgenic plants is already processed into the mature form, the neonates are exposed to a higher effective dose in the transgenic plants compared to the application of protoxin in the diet. This may resemble high dose conditions, where the immune induction mechanism is relatively ineffective. In contrast, toxin crystals taken up by neonates through artificial food will have to be processed and are not as active. This may represent low dose conditions, where the immune induction mechanism operates in a low dominant fashion.

Whatever the molecular basis, the similarities in the findings for *H. armigera* (Ma et al., 2005) and *E. kuehniella* (Rahman et al., 2004), where Bt-tolerance is associated with an elevated immune response and can be transmitted to offspring by a maternal effect, suggest that the capacity for species to develop Bt-tolerance through immune-related processes could be more common. These observations may have significant implications in the field. Although the observed levels of immune-related tolerance are low compared to those associated with receptor mutations, if low-level tolerance occurs through combinations of pre-existing alleles rather than rare mutations, it may play an important role as a first step to the development of higher level resistance.

The transmission of immune status and tolerance to subsequent generations by a maternal effect may slow the loss in genetic variation otherwise associated with selection. This could facilitate the emergence of more highly Bt-tolerant insects that in turn may survive in sufficient numbers under continued selection pressure to develop mutations that genetically fix the elevated immune status or alter receptor functions (Morin et al., 2003). Because the toxin expression level in Bt crops decreases gradually along with the development of the crop, conditions of low toxin dosage also can occur in the field (Liao et al., 2002). In this case, Bt resistance mechanisms with dominant traits may become relevant.

In conclusion, a number of observations suggest that an elevated immune status contribute to the observed Bt-resistance in a *H. armigera* strain. First, both gut extracts and plasma from the resistant strain displayed a higher melanization rate compared to the susceptible strain. Second, an inducible immune protein in the hemolymph plasma was identified as hexamerin, which acts as a storage protein and pro-coagulant (Scherfer et al., 2004). The protein bound to Cry1Ac and GalNAc-specific lectins to form an insoluble aggregate. Third, reciprocal crosses of resistant and susceptible insects revealed the transmission of the tolerance from one generation to the next by a maternal effect. As discussed for *E. kuehniella*, the most straightforward explanation for the observations is the incorporation of an immune-protein into the oocyte by an immune-induced female (Rahman et al., 2004). The protein can interact with embryonic tissues as an elicitor to induce the immune system of the neonate. By the time the neonate starts feeding, the insect already has an elevated immune status, thus increasing the chances of surviving the toxin. In turn, older larvae are able to survive the initial exposure to Bt for enough time on little or

no food for immune induction to occur. This may explain the puzzling observation that susceptibility to Bt-toxin decreases in later stages of larval development.

The fact that resistance is acquired in the embryo by an immune induction is an indication that the mutation in the resistant strain is not directly involved in altering the receptor-coding region. However, the finding that ALP is reduced in resistant larvae could point to altered phosphorylation in resistant larvae. Reduction of mALP at the BBMV could also account for the recessive effects of toxin binding, while the systemic reduction of phosphorylation could account for the semidominant effects of immune induction. While an unknown protein with the size range of a putative ALP shows reduced toxin binding, the inspection of several aminopeptidase N genes from the resistant strain did not reveal any mutational changes that could be responsible for the observed tolerance (Angelucci et al., 2002).

Appendix

1. Publications

1.1 Publications during the course of the current PhD study

1. Gang Ma, Douglas Hay, Dongmei Li, Sassan Asgari, Michael Kanost and Otto Schmidt (2005) Recognition and inactivation of LPS by lipophorin particles. Developmental and Comparative Immunology (in press, original publication)
2. Gang Ma, Muhammad Sarjan, Christopher Preston, Sassan Asgari and Otto Schmidt (2005) Mechanisms of inducible resistance against *Bacillus thuringiensis* endotoxins in invertebrates, Insect Science, 12: 231-240 (review)
3. Otto Schmidt, M Rahman, G Ma, U Theopold, Y Sun, M Sarjan and H Roberts, Mode of action of antibacterial proteins, pore forming toxins and biologically active peptides (2005), Invertebrate Survival Journal, 1: 82-90 (review)
4. Gang Ma, Harry Roberts, Muhammad Sarjan, Nicki Featherstone, Jelle Lahnstein, Ray Akhurst and Otto Schmidt, (2005). Is the mature endotoxin Cry1Ac from *Bacillus thuringiensis* inactivated by a coagulation reaction in the gut lumen of resistant *Helicoverpa armigera* larvae? Insect Biochemistry and Molecular Biology, 35: 729-739 (original)
5. Reineke A, Asgari S, Ma G, Beck M and Schmidt O. (2002). Sequence analysis and expression of a virus-like particle protein, VLP2, from the parasitic wasp *Venturia canescens*. Insect Mol Biol. 2002 Jun; 11(3): 233-9 (original)

1.2 LPS-mediated aggregation of lipophorin (manuscript for Developmental and Comparative Immunology)

Recognition and inactivation of LPS by lipophorin particles.

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Abbreviations: β-ME, 2-beta-mercaptoethanol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; IPTG, Isopropyl-beta-D-thiogalactopyranoside; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PPO, Prophenol oxidase; PTU, phenylthiourea; vWD, von Willebrand factor D domain;

The nucleotide sequence reported in this paper has been submitted to the GenBank™/EBI Data Bank: The sequence accession number of *Galleria mellonella* lipophorin is AY661711.

Key words: Lipopolysaccharide, lipophorin, coagulation, LPS binding protein, Galleria mellonella,

ABSTRACT

Lipophorin is the major lipid carrier in insects, but various observations indicate that lipophorin is also involved in immune reactions. To examine a possible role of lipophorin in defence reactions, we mixed hemolymph plasma from *Galleria mellonella* with LPS and noticed that lipophorin forms detergent-insoluble aggregates, while other plasma proteins are not affected. Lipophorin particles isolated by low-density gradient centrifugation retained LPS-induced aggregation properties, which suggested to us that these particles are able to recognise LPS and respond by forming insoluble aggregates. Antibodies against LPS-binding proteins, such as immulectin-2 and β-1,3-glucan binding protein, cross-reacted with proteins associated with lipophorin particles. To examine whether LPS-mediated aggregates inactivate LPS, we added LPS-lipophorin mixtures to purified lipophorin particles and monitored aggregate formation. Under these conditions lipophorin did not form insoluble aggregates, which indicates that lipophorin particles sequester LPS into non-toxic aggregates.

Invertebrate organisms with an open circulatory system possess effective coagulation pathways that mediate rapid aggregation of ubiquitous plasma proteins to prevent loss of body fluids and to inactivate intruding microbes and parasites [1,2]. The identification of coagulating proteins in different arthropod species suggests that a diverse group of proteins serve as pro-coagulants [2,3] that include lipophorin [4,5], hexamerin [5,6] and vitellogenin [7]. While functional properties of lipophorin as a lipid carrier are well characterised [8,9], the molecular role of apolipoproteins in immune functions is largely unknown. In addition to coagulation, immobilised apolipophorin I (apo I) molecules have been shown to alter hemocyte adhesion properties [10,11], but nothing is known about a possible mode of action. Likewise, lipophorin is known to be involved in detoxification of bacterial [12,13] and fungal toxins [14], but how these toxins are recognised and removed from circulation by lipophorin particles is not known.

One recognition and inactivation system involves plasma protein aggregation [4,5]. When hemolymph plasma fractions from *Hyalophora cecropia* pupae were mixed with bacteria a detergent-insoluble protein aggregate was formed on the bacterial surface, which was depend on calcium and the presence of LPS-specific sugar determinants [15]. One of the plasma components involved in the aggregate formation was hemolin, an immunoglobulin-like molecule from *H. cecropia* [16] and *Manduca sexta* [17]. Further studies suggested that while hemolin interacted with the bacterial surface [17,18], where it recognised the lipid A moiety of LPS [19], the aggregate formation [16] comprising unknown plasma components, depended on LPS-specific sugar residues [15]. This suggests that the recognition and cell-free effector reactions are two-step processes, where the recognition is Lipid A dependent and calcium-independent, whereas the formation of an insoluble complex is dependent on LPS-specific glycodeterminants and calcium.

Further understanding of this process requires the identification of the unknown plasma proteins involved in the complex formation. Moreover, given that hemolin is absent in many insects, the fundamental question is, what is the recognition process in general and how is the recognition of elicitors translated into the formation of a complex that inactivates the toxin. Here we show that LPS is recognised and removed from hemolymph plasma by an

aggregation process involving lipophorin particles in association with LPS-binding proteins (LBPs).

MATERIAL AND METHODS:

Insect culture. *Galleria mellonella* larvae were reared on an artificial diet at 25°C with a 10/14 h light/dark cycle.

LPS treatment of hemolymph. Hemolymph from six third-instar or two fifth instar larvae of G. mellonella was pooled in 1.5 ml PBS, and centrifuged at 3,000 x g for 5 min to remove hemocytes. 20 µl plasma (cell-free hemolymph) was mixed with the same volume of LPS (20 µg/ml) and incubated at RT for various time periods. In a similar experiment plasma was mixed with LPS (20 µg/ml) together with β-mercaptoethanol (2mM final concentration) and incubated under reducing conditions at RT for various time periods. The mixture was centrifuged at 10000 x g for 10 min. An aliquot from supernatants for each time period was separated on 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue stain-R250 for 30 min then destained overnight. The identity of the apolipophorin I protein band at ca 230 kDa was confirmed by Western blots using antibodies against a cloned G. mellonella protein domain with similarities to van Willebrand factor D domain (vWD) and antisera against lipophorin from blowfly (Lucillia cuprina).

Expression and purification of the G. mellonella vWD-like domain. A DNA fragment containing the *G. mellonella* vWD domain was sub-cloned from a partial cDNA clone, which was previously isolated from an immune screen [20]. The vWD-like domain of *G. mellonella* was further analysed by the method previously described [21]. Two primers, which flank the region of interest, were designed with the introduction of a *Bam*HI restriction site at 5' end and the *Hind*III at the 3' end. The primers were, GmvWFBamHI-F:
5'-CGCGGGATCCATTCGATGAAATTCCTGC-3' and GmvWFHindIII-R:
5'-GCGCAAGCTTACGCGGGATCCATTCGATGAAATTCCTGC-3'. The PCR reaction produced the expected amplified cDNA product (471 bp in size), which was cut from the gel and purified with the Qiagen system and ligated into the pGEM-T easy vector (Promega). The insert was confirmed by further sequencing.

The cloned insert was digested with *Bam*HI and *Hind*III and ligated into the pQE31 expression vector (Qia express®), which was pre-cut with the same restriction enzymes. The ligation mixture was used to transform M15 competent cells. After PCR identification, the bacterial colony containing the correct insert was grown and induced with 1 mM IPTG. Expression of the fusion protein containing 6×His residues was confirmed by SDS-PAGE and Western blot analysis using anti-His antibodies (Sigma). Since the protein was found to be in the insoluble fraction, the recombinant protein was purified under denaturing conditions using Ni-NTA beads as described by the manufacturer (Qiaexpress®).

Antiserum against vWD-157. Antibodies against the vWD-like domain from G. mellonella was made as described [22]. The purified fusion protein was separated by SDS-PAGE and visualised by water-based Coomassie staining. The band was cut and ground in PBS (500µl) to which complete Freund's adjuvants were added (500 µl), and injected subcutaneously into rabbits. Boost injections were performed twice in the third and fifth week respectively. Antisera against imaginal disc growth factor (IDGF) from Pieris rapae was used as described [23]. Antibodies against arylphorin was a kind gift from K. Schaller (Wűrzburg, Germany).

Low-density gradient centrifugation. Hemolymph from ten third and fourth instar larvae were collected in 0.5ml PBS (with and without 2.5mM EDTA) and phenylthiourea (PTU). Hemocyes were removed by centrifugation at 3000xg at 4°C for 15 minutes in an Eppendorf centrifuge and plasma diluted to 15ml. O.433 g/ml KBr in PBS was added and the solution overlaid with 0.9% NaCl/PTU solution (with and without EDTA) and centrifuged overnight at 24,000rpm at 10°C in a SW32Ti rotor (Beckman). The gradient was collected in fractions of 1ml each and stored at 4°C. For SDS-PAGE and bioassays fractions were used directly or after dialysis and diluted 5 times using PBS (with or without EDTA).

LPS bioassay using lipophorin particles. We used the detergent-insoluble complex formation of lipophorin in the presence of elicitors [4,20], as a response

indicator to LPS. Plasma or gradient fractions were mixed with different concentrations of LPS (steps of half concentrations starting from 500 µg/ml to 2 µg/ml) left at 37oC for 45 minutes and centrifuged at full speed in an Eppendorff centrifuge for 10 minutes. Loading buffer was added to the supernatant and heated at 65°C for 5 minutes before loading on a 10% polyacrylamide gel. SDS-PAGE and Western blots were performed as described earlier [5,6]. Antiserum against immulectin-2 and β-1,3-glucan-binding protein were used as described [24].

RESULTS

Lipophorin aggregate formation

Preliminary experiments and published data [4,20] suggested that lipophorin forms detergent-insoluble aggregates in the presence of elicitors. While this precludes biochemical analysis of aggregate components, it should be possible to detect the absence of apolipoproteins from plasma supernatants, even if the aggregates in the pellet are insoluble. To identify the plasma components that interact with elicitors, such as LPS, we analysed the remaining soluble plasma components on SDS-PAGE and Western blots. Under these conditions, apolipophorin was selectively removed from the supernatant, whereas other major plasma components remained in solution (Fig. 1A). When the corresponding pellets were treated with various chaotropic solutions, such as SDS and guanidylchloride, the aggregates did not dissolve and the proteins missing from the supernatant were not recovered in a soluble form (not shown).

This reaction can be inhibited by various treatments and inhibitors of coagulation. Apart from EDTA (see below), the presence of reducing agaents, such as ß-mercaptoethanol (ß-ME) in particular concentrations prevented aggregation into detergent-insoluble complexes (Fig. 1B).

Purified lipophorin particles

To investigate LPS-mediated lipophorin aggregation in the absence of hemolymph plasma, we isolated lipophorin particles using low-density

gradient centrifugation. Under these conditions the major plasma proteins not associated with lipids were separated from lipid-containing proteins, which moved into the low-density regions of the gradient. Major lipoproteins, such as lipophorin particles form several peaks, depending on the lipid content and proteins associated with it, with a major band visible to the eye by a yellowish colour. To examine the distribution of hemolymph proteins along the gradient, we analysed aliquots of the gradient fractions on SDS-PAGE and corresponding Western blots using antiserum against various plasma proteins (Fig. 3). Whereas plasma proteins, such as prophenoloxidase (PPO) and arylphorin, were concentrated in the high-density region of the gradient with a peak in fractions (5-7), both proteins became undetectable in fractions 14-16 (Fig. 3). In contrast, apolopophorin I and II are not detected in high density fractions but became visible in fractions 16 and peaked in fractions 18-20, where it is visible as a yellowish coloured band in the gradient (dense lipophorin particles). One or more additional lipophorin peaks are detected in the low-density regions of the gradient (fractions 24-30), which may represent other lipid carriers or light lipophorin particles.

Interestingly, some proteins found in the plasma fractions also co-purified with lipophorin particles. For example, the chitinase-like lectin with similarity to imaginal growth factors co-purified with lipophorin particles (Fig. 3), which could indicate an association with lipophorin particles.

When Western blots containing aliquots from the gradients were analysed using antibodies against the LPS-binding proteins immulectin-2 and the β-1,3-glucan binding protein, both proteins were detected in the lipophorin fractions, in addition to lipophorin-free plasma (Fig. 2). In the presence of EDTA the amounts of LPS-binding proteins are significantly reduced but are still detectable. This suggests that the two LPS binding protein are associated with lipophorin particles and are gradually removed from particles in the presence of EDTA.

LPS-mediated lipophorin particle aggregation

To examine whether purified lipophorin particles retain the ability to recognise LPS and aggregate into detergent-insoluble complexes, we added LPS to

gradient fractions containing lipophorin particles. Under these conditions LPS-mediated aggregation was detected in freshly prepared fractions in a concentration-dependent fashion (Fig. 3A). When fractions were left for a few days, lipophorin disappeared into detergent-insoluble fractions even in the absence of LPS. To keep lipophorin under stable conditions, plasma was prepared in the presence of EDTA and fractions kept or dialysed with EDTA. Under these conditions lipophorin fractions were stable for up to a week and responded (albeit less vividly) to LPS (not shown). After more than a week in the presence of EDTA lipophorin fractions did not respond and both apolipophorins remained in solution in the presence of LPS (Fig, 3B). This suggests that the ability of lipophorin particles to recognise LPS and aggregate into insoluble complexes is Ca-dependent and can be abolished by EDTA.

LPS inactivation by lipophorin particles

Since intact lipophorin particles are sensitive and react in a concentration-dependent fashion to LPS, we asked whether LPS is still reactive or whether this reaction abolished the elicitor function of LPS. We therefore performed two subsequent assays, where the outcome of the first reaction was tested for LPS to function as an elicitor in a second reaction. We used lipophorin-free plasma fractions, which contained LPS-binding proteins (Fig. 2), in a first reaction as a control. LPS was added to fractions 8 and 20 and the mixture was added to fraction 20 and examined under SDS-PAGE. When the LPS-lipophorin mixtures were added to lipophorin particles, the apolipophorins remained soluble, which indicates that the mixture did not contain any active LPS (Fig. 4). In contrast when lipophorin plasma proteins were mixed with LPS, the LPS retained its function as an elicitor for the aggregation of lipophorin, except in very high LPS concentrations, where plasma proteins seem to inactivate LPS by an unknown process.

Discussion

Here we show that lipophorin particles are able to recognise and inactivate LPS in a concentration dependent fashion. The ability to recognise LPS is due to an association of LPS-binding proteins (LBPs), such as immulectin-2 and a ß-1,3-glucan-binding protein [24], with lipophorin particles. Lipophorin particles depend on these pattern recognition molecules to form detergent-insoluble complexes. Prolonged exposure of particles to EDTA dissociated pattern recognition molecules from lipophorin particles, which become insensitive to LPS and remain stable in LPS containing solutions.

We used the assembly into detergent-insoluble lipophorin complexes [4] as an indicator for LPS-mediated responses by lipophorin particles. The observation that attachment of LBPs to lipophorin particles is abolished in the presence of EDTA, which renders the particles unresponsive to LPS, suggests that LPS-binding to LBPs is a precondition but not sufficient for the inactivation of LPS. This was confirmed by two consecutive assays, where LPS was first mixed with lipophorin free plasma proteins, which contain LBPs, and subsequently added to purified lipophorin particles. Under these conditions lipophorin particles reacted to LPS, whereas no reaction was observed when LPS was mixed with lipophorin particles first and the mixture added to a fresh batch of lipophorin particles.

While it is known that LPS binds to lipoproteins in vertebrate cell-free systems [25,26] and that lipid particles are able to inactivate LPS [12,27], the mechanism of how LPS interacts with lipophorin and how lipophorin can make LPS inaccessible to further interactions is not known. Our observations together with previous data on bacterial recognition by hemolin [15-19] suggest that the recognition of LPS and subsequent cell-free effector reactions are two-step processes, where the recognition is a Lipid A-dependent reaction, whereas the formation of an insoluble complex is dependent on LPS-specific glycodeterminants. This raises the question about the nature of the observed detergent-insoluble complex that is able to shield LPS from further interactions.

The fact that LPS forms aggregates that are considered the biologically active forms of LPS [28] could indicate that multiple proteins interact with LPS

molecules. This is reminiscent of the multimerization and oligomerization of lectins and pore-forming toxins interacting with pro-coagulants, including lipophorin [20,29], which may be a precondition for toxin inactivation [30]. While pro-coagulants form fibre-like chains [7], this is an implausible structure to shield the toxin from further interactions with other proteins. Alternatively, assembly of lipophorin particles into globular structures are the result of oligomeric interactions, which provide cage-like coagulation products, where the lipid moiety forms a protective layer that separates the toxin from interactions with outside molecules.

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Figure legends:

Fig. 1 *G. mellonella* hemolymph plasma supernatants from six third instar larvae after forming a complex with LPS at various time periods and under different incubation conditions. Commassie stained gels after separation of protein extracts using SDS-PAGE. **A**) Plasma incubated with 20ug/ml LPS at various time periods. Note the reduction of the apolipophorin I protein band at around 230 kDa (arrow). Larger protein aggregates are visible in LPS-treated fractions some of which are not entering the gel. These aggregates contained lipophorin, which was confirmed by Western blots using several antisera against lipophorin. **B**) Plasma incubated with 20ug/ml LPS and β-mercaptoethanol (2mM final concentration) at various time periods. Note the presence of apolipophorin I molecules at the correct position (arrow). M: Prestained SeeBlue™ molecular weight markers.

Fig. 2 Protein distribution of low-density gradient centrifugation of plasma proteins, **A**) Coomassie blue staining of SDS-PAGE separated aliquots of fractions 14-18 and corresponding Western blots using antibodies against recombinant vWD domain in apolipophorin I, arylphorin and IDGF. Note that arylphorin, a typical plasma protein without lipid-associations is visible in fractions below 14, whereas apolipophorin is visible in fractions 16 and above. IDGF is present in both regions of the gradient and may be associated with lipophorin particles. **B**) Co-purification of LPS-binding proteins, such as immulectin-2 and ß-1,3-glucan-binding protein with lipophorin particles after low-density gradient centrifugation of plasma proteins in PBS buffer. **C**) In the presence of EDTA the amounts of LPS binding protein in lipophorin fractions is significantly reduced but still visible.

Fig. 3 LPS aggregation assay using purified lipophorin particles (fraction 18) with increasing amounts of LPS. A) Low-density gradient centrifugation of plasma protein in PBS and aliquots of fractions mixed with increasing amounts of LPS (between 20 and 100 ng). The reaction was performed within two days after centrifugation. Lipophorin particles are not detected after SDS-PAGE and may have formed spontaneous aggregates. **B**) Low-density gradient centrifugation of plasma protein in PBS, containing EDTA and

aliquots of fractions mixed with increasing amounts of LPS (between 10 and 100 ng). In contrast to PBS fractions, which disappear, prolonged exposure of lipophorin particles to EDTA, makes lipophorin insensitive to LPS. After more than a week in EDTA buffer, LPS addition has no effect on lipophorin aggregation.

Fig. 4 LPS is inactivated by lipophorin particles. **A**) LPS elicitor function was examined after interacting with lipophorin particles. First, LPS was added to lipophorin particles (fraction 18) and aliquots analysed on SDS-PAGE. This shows that aggregates are formed at high LPS concentrations. Aliquots from the same reaction were added to a fresh aliquot of lipophorin particles and analysed on SDS-PAGE. This shows that lipophorin molecules are not affected in any of the LPS concentrations indicated that induced lipophorin aggregation in the previous reaction. **B**) In contrast when LPS was added to lipophorin-free plasma (serum), and aliquots analysed on SDS-PAGE, no insoluble complexes where formed, although the serum contained pattern recognition molecules. When aliquots from the same reaction were added to a fresh aliquot of lipophorin particles and analysed on SDS-PAGE, lipophorin reacted with LPS from the first reaction. This suggests that LPS is still active to form detergent-insoluble complexes after reacting with lipophorin-free plasma.

Fig. 1

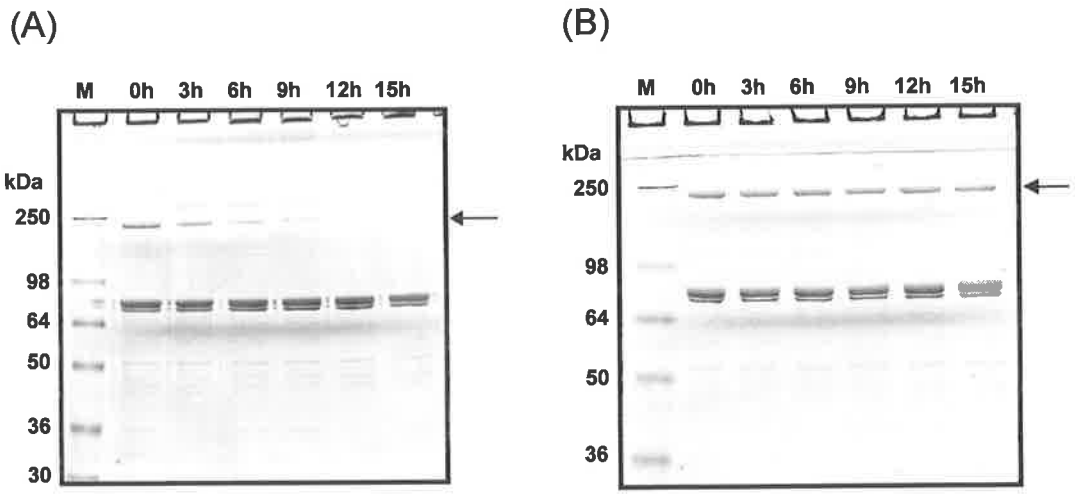


Figure 2

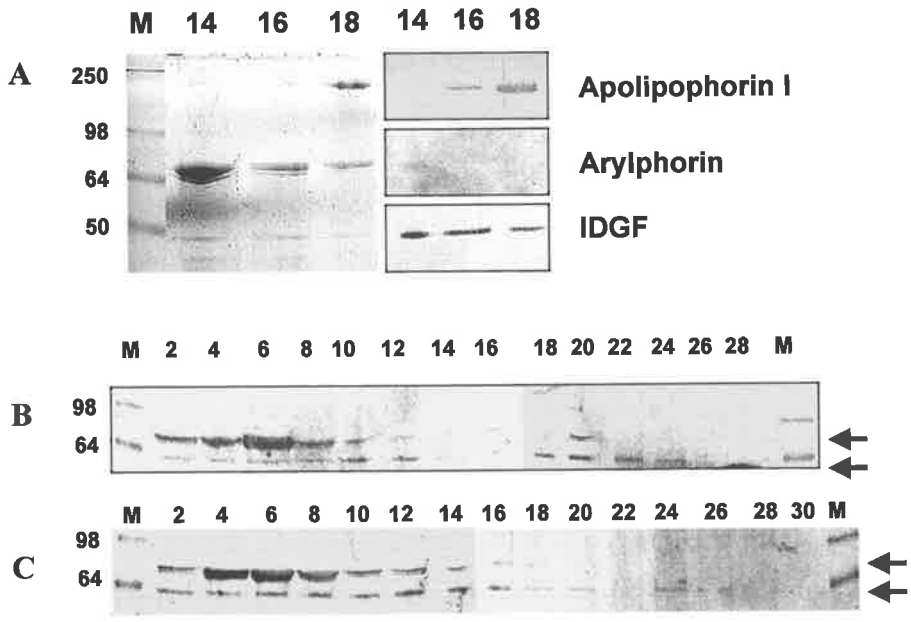


Figure 3:

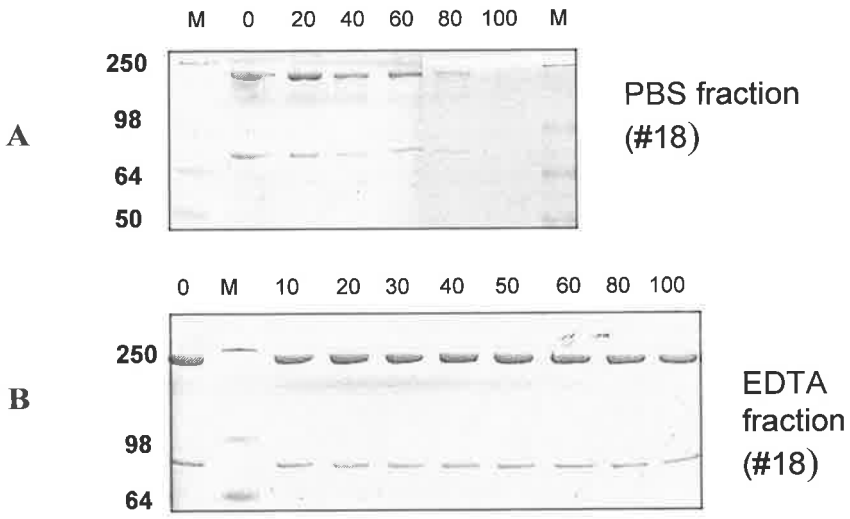

A
M 0 20 40 60 80 100 M
250
98
64
50
PBS fraction
(#18)
B
0 M 10 20 30 40 50 60 80 100
250
98
64
EDTA
fraction
(#18)

Figure 4A

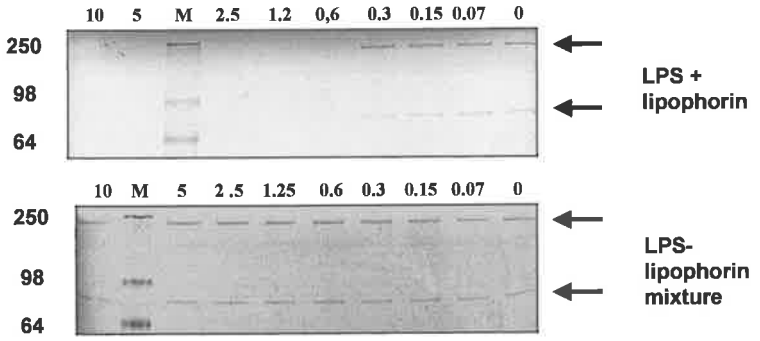
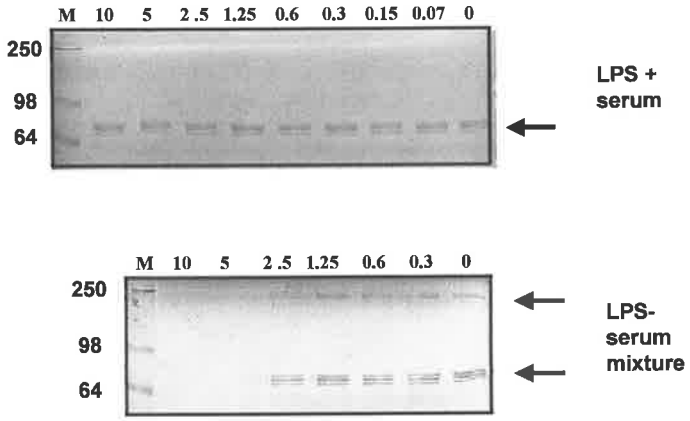


Fig. 4B



2. Abbreviations

Ala	alanine
ALP	alkaline phosphatase
APN	aminopeptidase N
Arg	arginine
Asn	asparagine
BBMV	brush border membrane vesicle
BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
bp	base pair
Ca	Casium
CBBR	Coomassie brilliant blue stain R-250
CNBr	cyanogen bromide
dNTPs	deoxynucleotide triphosphatase
DDT	dithiothreitol
DOPA	dihydroxyphenylalanine
ELLA	enzyme-linked lectin assay
Gal-T	galactotransferase
GalNAc	galactosylamine
GlcNAc	glucosylamine
Gln	Glutamine
Gly-T	glycosylatransferase
Glu	glutamic acid
GPI	glycosylphosphatidylinositol
GSP	gene specific primer
His	histidine
HPLC	high performance liquid chromatograph
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilo Daltons
LB	Luria Bertani
LPS	lipopolysaccharide
Lys	lysine

M	molar
μM	micro molar
μg	microgram
μl	microliter
MALDI-TOF	Matrix assisted laser desorption ionisation-time of flight
Model I	receptor inactivation Bt resistance mechanism
MOPS	3-[N-morpholino] propanesulfonic acid
MPI	mannose phosphate isomerase
NC	nitrocellulose membrane
NCBI	National centre for biotechnology information
Ni-NTA	Nickel-nitrilotriacetic acid
ORF	open reading frame
^{32}P	α-^{32}P-labelled deoxycytosine triphosphate
PAGE	poly acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPO	prophenoloxidase
PM	peritrophic matrix
PVDF	polyvinylidene diflouride
RACE	rapid amplification of cDNA ends
RNAi	RNA interference
RP-HPLC	reverse phase high performance liquid chromatograph
RT	room temperature
RT-PCR	reverse transcript polymerase reaction
TBST	tris buffered saline with tween
vWD	von Willebrand factor D domain
SDS	sodium dodecyl sulphate
SSC	sodium sodium citrate
TAE	Tris-acetate/EDTA buffer
Tris	Tris-hydroxymethyl-aminomethane
X-gal	5-bromo-4-chloro-3-indolyl-β-D galactopyranoside

3. Solutions, buffers, and diet ingredients

3.1 Protein preparation and analysis

PBS

10x Phosphate Buffered Saline, pH 7.4 (0.2 M phosphate, 1.5 M NaCl)

2.28 g NaH_2PO_4 (mw=120); 0.038M)

-or- 2.62 g $NaH_2PO_4(H_2O)$ (mw=137.99; 0.038M)

11.5 g Na_2HPO_4 (mw=141.96; 0.162 M)

43.84 g NaCl

pH to 7.4 Final volume to 500 ml with water

PBS-Tween (washing solution) (PBS plus 0.05% Tween-20, 0.02% sodium azide)

400 ml 10x PBS

8 ml 10% NaN_3 (azide)

2 ml Tween-20

Final volume to 4 L with H_2O

4 X SDS-PAGE sample loading buffer (normal) (10 ml)

0.5M Tris-HCl,	5 ml	1M Tris-HCl, pH 6.8
40% glycerol	4 ml	100% glycerol
β- mercaptoethanol	0.8 ml	β-mercaptoethanol
Bromophenol blue (BPB)	0.04 g	
SDS	0.8 g	

Aliquot and freeze at –20°C

10 X SDS-PAGE running buffer (2 litre)

250 mM Tris-base	60.57 g
14.4 % Glycine	288 g
1% SDS	20 g

Add MQ water to 2 litres and adjust the pH around 8.6.

Towbin transfer buffer (1 litre)

25 mM Trisbase	3.03 g
192 mM glycine	14.4 g
20% methanol	200 ml

Adjust volume to 1 litre with MQ water, store at 4˚C

Coomassie brilliant blue R-250 stain buffer (acid-soluble) (400 ml)

45% methanol	180 ml methanol
9% acetic acid	36 ml acetic acid
0.25% (CBBR-250)	1 g CBBR-250

Add MQ water to 400 ml and mix well

Comassie R-250 destain buffer (acid-soluble) (400 ml)

12% methanol	48 ml methanol
7% acetic acid (glacial)	28 ml acetic acid glacial

Add water to 400 ml

0.1% Ponceau S dye in 1% aqueous acetic acid

Ponceau-S destaining buffer

1% acetic acid

Comassie R-250 stain buffer for PVDF

0.025% Coomassie® Blue R-250 dissolved in a 40% MeOH solution

Comassie R-250 destain buffer for PVDF

50% methanol

NC block buffer for lectin and Cry1Ac

0.1-3% BSA in TBS or PBS buffer

Peroxidase developing buffer (antibody or lectin)

10 mM Tris, pH 7.5

0.1% H_2O_2

A grain of diaminobenzide as substrate

NC Blocking buffer I

16 g milk powder

200 ml PBS

400 µl 10% sodium azide, store at 4˚C

NC blocking buffer II

10 g milk powder

30 ml 1 M NaCl or 6 ml 5 M NaCl

10 ml 1M Tris-HCl pH 7.5

800 µl 10% sodium azide, store at 4˚C

Washing Buffer (1 X TBST in 1 litre)

20 mM Tris.HCl	Trisbase	2.4 g
500 mM NaCl	NaCl	29.2 g
0.05% (v/v) Tween-20	Tween-20	500µl
0.2% (v/v) Triton X-100	Triton X-100	2 ml

Adjust pH to 7.5 with ca. 1.8 ml HCl

Alkaline phosphate developing buffer (antibody)

100 mM NaCl

5 mM $MgCl_2$

100 mM Tris-HCl pH 9.5

Substrates for developing alkaline phosphate reaction

BCIP: 5-Bromo-4-Chloro-3-indolyl phosphate

NBT: Nitro blue tetrazolium

Peroxidase developing buffer (antibody or lectin)

10 mM Tris, pH 7.5

0.1% H_2O_2

Diaminobenzide

p-Coumaric acid stock solution

11.5 mg in 10 ml of DMSO

Luminal (5-amino-2, 3-1,4-pahatalazinedion)- H_2O_2 stock solution

2.5 mg luminal in 10 ml 0.1 M TrisHCl pH 6.8

Add 6 µl of 30% H_2O_2

Antibody making buffer for injection

Complete

Incomplete

Antibody elution from Western blot

Washing solution

10 ml of PBS

50 µl of Tween 20

Elution Buffer	**EB (10ml)**
0.05 M Glycine-HCI (pH2.3)	0.5 ml from 1 M
0.05 M NaCI	0.5 ml from 1 M
0.5 % (v/v) Tween 20	50 µl
100 µg/ml BSA	0.001g

Neutralizing solution

Na_2PO_4 to a final concentration of 50 mM

5 µl 1 M/100 µl of EB

3.2 Competent cell

TFB 1

100 mM RbCl,

50 mM $MnCl_2$,

30 mM Kac,

10 mM $CaCl_2$,

15% glycerol, pH 5.8 sterile filtered

TFB 2

10 mM MOPS,

10 mM RbCl,

75 mM $CaCl_2$,

15% glycerol, pH 8.0 autoclaved

3.3 DNA ligation, transformation, and expression

IPTG (500mM)

119 mg per ml autoclaved water

Weigh out approximately this amount; note the amount weighed and dissolve in the appropriate volume of autoclaved water. Store at 4°C.

L Broth Agar

1 L of L Broth

15 g agar

Autoclave

LB medium

10 g bacto-tryptone

5 g bacto-yeast extract

5 g NaCl per litre

3.4 RNA extraction and Northern blot

"Solution D"

Mix 50 ml stock solution D with 0.36 ml 2-mercaptoethanol

"Stock solution D":

250 g guanidium thiocyanate

293 ml DEPC-treated water

17.6 ml 0.75 M sodium citrate, pH 7

26.4 ml 10% sarcosyl at 65˚C

50% Glycerol

25 ml 100% glycerol

25 ml water

Autoclave. Concentrated glycerol is quite viscous. Be sure all of it has been transferred from the stock bottle.

Deionized Formamide

Mix 10 ml formamide with 0.5 g mixed bed resin (Sigma; Amberlite MB-1) gently for 30 minutes at room temperature. Filter through a Whatman #1 filter to remove resin. Aliquot 950 µl into a 1.5 ml microfuge tube to make 1 ml formamide dye mix for sequencing. Store at 4°C.

10x MOPS Running Buffer

41.96 g MOPS free acid (0.2 M, pH7.0)

4.1 g sodium acetate (50 mM)

10.0 ml 0.5 M EDTA (5 mM, pH 8.0 stock)

Adjust pH to 7.0 with NaOH. Volume to 500 ml with water. Wrap in foil (light sensitive) and autoclave.

Sample Loading Buffer

For around 20 µg of total cellular RNA or 1-2 µg of poly-A RNA,

final volume to 5 µl with water.

Add 2 µl 10x MOPS running buffer,

3.5 µl formaldehyde and 10 µl of deionized formamide.

Heat 10 - 15 minutes at 65°C.

Place on ice and add 2 µl of stop dye.

20X SSC

175.4 g NaCl (3 M)

88.2 g sodium citrate dihydrate (0.3 M pH 7.0)

Adjust pH to 7.0 with HCl. QS to 1 L with water. Autoclave.

Northern blot block solution

20 X SSC	3 ml
50 X Denhardt's	1 ml
10% SDS	0.4 ml
H_2O	5.6 ml
Sperm DNA (10 mg/ml)	200 µl

Northern blot washing buffer

	First wash 2X SSC +0.1% SDS	Second wash 0.2X SSC +0.1% SDS
20 X SSC	40 ml	4 ml
Water	356 ml	392 ml
10 % SDS	4 ml	4 ml
Final Vol	400 ml	400 ml

3.5 Insect rearing

3.5.1 *H. armigera* diet ingredient

Diet mixture	
Soya Flour	130g
Wheat Germ	60g
Brewers Yeast	53g
Ascorbic Acid	3.3g
Sorbic Acid	1.7g
Nipagen	3.3g
Agar Mixture	
Agar	20g
Water	300ml

3.5.2 Preparation of *H. armigera* diet

Pre-weighed dry ingredients (see Appendix) were mixed with 0.7 litre of fresh boiled Milli-Q water and blend thoroughly. Next step was to combine agar and cold water in a tall beaker and microwave on high level for 3 minutes. The mixture of dry ingredients was mixed with agar mixture and top up to 1600ml using cold water that has been previously boiled. Blended until well combined. The temperature of the diet was below 63°C before adding preservatives. The mixture was then poured into trays. The resultant artificial diet can be stored for up to a month (L. Bird, personal communication).

3.5.3 *H. armigera* rearing conditions

Adult female and male were selected for mass reciprocal crosses. Once the pupae were sexed according the method of (Nacrajan et al., 1979), they were sterilised in

5% White King bleach for 10 min, and placed separately in a pupal container lined with towel paper. The container was placed in the insectary at 25°C and 45% relative humidity with a 14/10 (L/D) h light/ dark cycle. Reciprocal crosses were made between females and males from the two strains based on the designed experimental requirements. Once the adult emerged (normally, female pupae come out 1-2 days earlier than males, with the colour of female adults relatively darker than that of males), they were placed in a mesh-covered container. The 3-day-old female adults met with 1-day-old males to yield fertilised eggs. The adults were maintained under the same condition as that of pupae. The feeding solution for adults was made up by mix of 20 g honey, 20 g sugar and 6 g ascorbic acid per litre (Akhurst et al., 2003). The solution, which was kept in a small bottle with a cotton dental roll inserted through the lid to deliver the solution to the insect, would help female adults lay healthier eggs on the cloth. The top cloths were changed daily to get the fresh eggs. The fresh eggs were sterilised in 5% White King bleach for 10 minutes, then dried on a paper towel. They hatched two days later in plastic bags. Placed the neonate on a plate, which contained the artificial diet.

3.5.4 Sex determination of *H. armigera* pupae

Bt toxin bioassay was used to elucidate the inheritance of Bt resistance in *H. armigera*. In this method, the first step is to set up genetic crosses. To make crosses, well-formed pupae were selected and their sex determined according to the method of (Nacrajan et al., 1979). In brief, the distance between the genital pore and anal pore, and the inverted "V" was used for separation of the two sexes. The distance between genital pore and anal pore of the female pupae is longer than that of male counterpart

(about two-fold). Furthermore, the inverted "V" line around the genital in female pupae is also a good indicator of sex (Fig. 1-13).

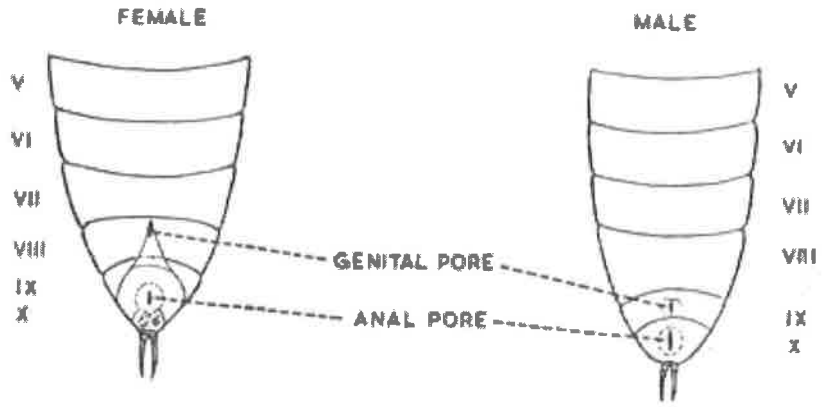


Fig. 5-1 Abdominal portion of *H. armigera* (Hunner) pupae showing difference in sexes (Nacrajan et al., 1979).

3.5.5 *G. mellonella* diet ingredient

1 packet of HEINZ® high protein baby cereal,

100g of wheat germ,

1 g of torula yeast,

0.15 g of nipagin,

5 capsules multi-V tablets

100 ml of honey

100 ml of glycerol

4 Antibodies used for the project

Antibodies against the proteins of interest

Name	Character	Organism	Source
92-1	Anti Gly-T antibodies	*C. elegans*	(Griffitts et al., 2001)
93-1	Anti Gly-T antibodies	*C. elegans*	(Griffitts et al., 2001)
mALP	Anti mALP antibodies	*B. mori*	(Itoh, Per. Comm.)
sALP	Anti sALP antibodies	*B. mori*	(Itoh, Per. Comm.)
Arylphorin	Anti Arylphorin	*Calliphora*	(Scheller, Per. Comm.)
vWD	AntiGmvWD-157	*G. mellonella*	(Ma, unpublished data)
Hemo C	Anti Hemocyanin C	*H. armigera*	(Ma et al., 2005)
Hemo M	Anti Hemocyanin M	*H. armigera*	(Ma et al., 2005)
Pro-coagulant	Anti coagulant	*G. mellonella*	(Li et al., 2002)
GBP	Anti β-Glucan binding protein	M. sexta	(Kanost, Per. Comm)
IML-2	Anti IML-2	M. sexta	(Yu and Kanost, 2003)

Antibodies for Western blot development

Anti-Rabbit IgG (whole molecules), alkaline phosphatase conjugate, developed in goat (Sigma)

Anti-Rabbit IgG (whole molecules), peroxidase Conjugate, developed in goat (Sigma)

Monoclonal Anti poly-histidine Clone His-1, alkaline phosphatase conjugate, purified mouse immunoblobuin (Sigma)

5. GeneBank accession numbers of DNA sequences

Name of gene	Species	Number	Length (bp)
Hexamerin	*H. armigera:*	AY661710	2442
Apolipophorin	*G. mellonella*	AY661711	4546
VWD domain	*P. xylostella*	AY661712	336
VWD domain	*H. armigera*	AY661713	306
VLP2	*Venturia canescens*	AF410773	1928

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